“COMPARISON OF IMMUNOCHROMATOGRAPHY WITH RT-PCR FOR DETECTION OF ROTAVIRUS IN FECAL SAMPLES”

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BRANCH IV MICROBIOLOGY

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This is to certify that the Dissertation **"COMPARISON OF IMMUNOCHROMATOGRAPHY WITH RT-PCR FOR DETECTION OF ROTAVIRUS IN FECAL SAMPLES"** presented herein by **Dr. G.MANJULA** is an original work done in the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D. (Branch IV) Microbiology under my guidance and supervision during the academic period of 2010 - 2013.

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Tirunelveli Medical College,
Tirunelveli - 627011.

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Dr. N. Palaniappan M.D.,
Guide,
Professor and Head ,
Department of Microbiology,
Tirunelveli Medical College,
Tirunelveli –11.

Dr. N. Palaniappan M.D.,
Professor and Head,
Department of Microbiology,
Tirunelveli Medical College,
Tirunelveli –11.

TIRUNELVELI MEDICAL COLLEGE

TIRUNELVELI,

STATE OF TAMILNADU, INDIA

PIN CODE:627011

Tel: 91-462-2572733, 2572734 Fax: 91-462-2572944

Estd:1965

Under the Directorate of Medical Education, Government of Tamilnadu.

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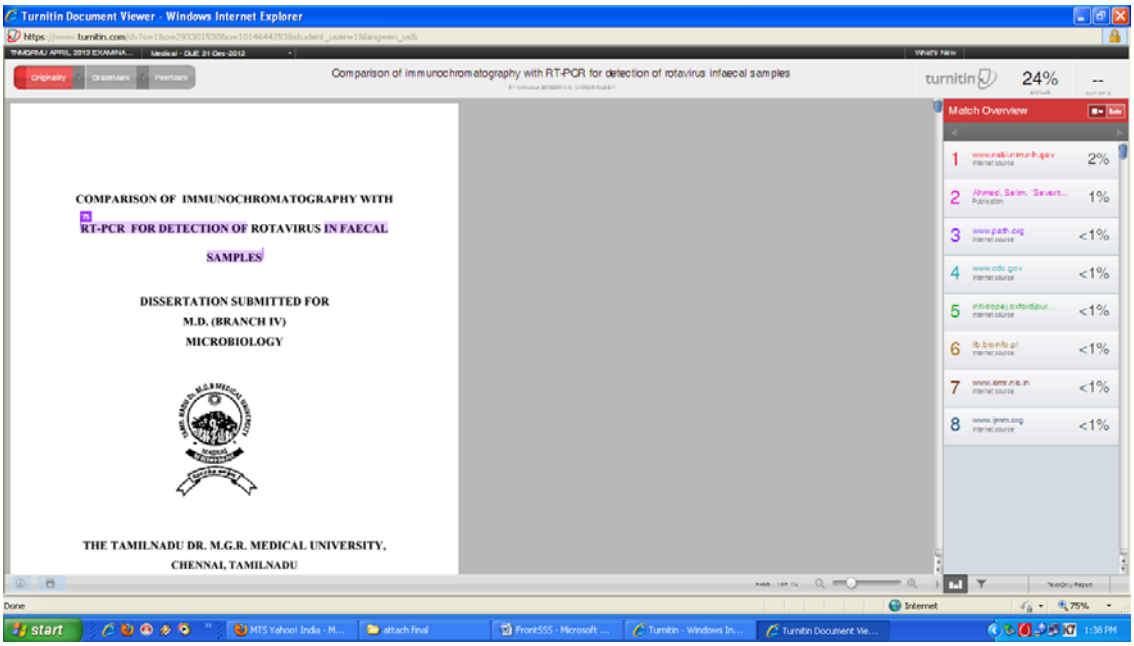
Tirunelveli-11.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CHENNAI, TAMILNADU

DECLARATION

I solemnly declare that the dissertation titled "**COMPARISON OF IMMUNOCHROMATOGRAPHY WITH RT-PCR FOR DETECTION OF ROTAVIRUS IN FECAL SAMPLES**" is done by me at Tirunelveli Medical College hospital, Tirunelveli.

The dissertation is submitted to The Tamilnadu Dr. M.G.R.Medical University towards the partial fulfillment of requirements for the award of M.D. Degree (Branch IV) in Microbiology.

Place: Tirunelveli
Date:

Dr. G.MANJULA,
Postgraduate Student,
M.D Microbiology,
Department of Microbiology,
Tirunelveli Medical College
Tirunelveli.

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ABBREVIATION

WHO	-	World Health Organisation
UNICEF	-	United Nations Interanational Children's Emergency Fund
ICT	-	Immunochromatographic Test
ELISA	-	Enzyme Linked Immuno Sorbent Assay
PCR	-	Polymerase Chain Reaction
RT-PCR	-	Real – Time PCR
Q-PCR	-	Reverse Transcriptase PCR
Ctvalue	-	Crossing Threshold Value
dNTP	-	Deoxy Ribonucleotide Triphosphate
FAM	-	6-Carboxy Fluorescein
BHQ	-	Black Hole Quencher
UIP	-	Universal Immunisation Programme

CONTENTS

1. INTRODUCTION

Diarrhoeal diseases have been recognized in humans since antiquity. Diarrhoeal diseases are one among the most common illnesses in infants and young children all over the world. Acute diarrhoeal diseases are the major cause of mortality and morbidity in children especially in developing countries like India. They are one among the six major causes of childhood mortality accounting for 18% of the deaths in children less than five years, with highest mortality in developing countries.[1]

1.1 Prevalence

1.1.1Global prevalence

Around one million children below the age of five die due to diarrhoeal diseases.[2] China, Democratic Republic of the Congo, Ethiopia, India, Nigeria, and Pakistan are the six countries responsible for more than 50% of these deaths occurring globally. Rotaviruses are the single most important etiological agent causing severe diarrhoeal disease in children less than five years of age in both industrialised and resource poor nations ,responsible for 30-50% of these diseases.[3]

Group A rotaviruses (HRV) are the major causative agent of childhood diarrhoea worldwide followed by enteric adenoviruses types 40 and 41 and other viral agents causing diarrhea. [4]

Every year 1000 lakhs of rotavirus induced diarrhoea in children are managed at home, 250 lakhs attend outpatient department, 20 lakhs admitted in hospitals and 3.52–5.92 lakh deaths occur below the age of five .Almost every child will have an episode of rotavirus gastroenteritis by the age of five , one of five children attends outpatient department and one of sixty requires inpatient management. Around one in two hundered and ninety three children die due to rotaviral diarrhoea.Among the mortality caused by rotavirus in the under five age group,eighty two percent occurs in socioeconomically poor countries. [5] Because of the high number of rotavirus deaths in under five age group , especially in resource poor countries,emergency steps have to be taken to reduce the mortality.

1.1.2 Indian scenario

Among the causes of death in socioeconomically poor nations,diarrhoea stands third. Around 98,621 rotavirus-induced diarrhoeal deaths have been recorded India in 2008, which is about 22% of global deaths. Nigeria recorded about 41,000 deaths, the second worst-hit country had less than half of mortality as compared to India. [6]

India has the highest death toll of more than 100,000 followed by more than 30,000 deaths in China and more than 25,000 in Pakistan.[4]

The proportion of all diarrhoea hospitalizations representing severe cases caused by rotavirus has been evaluated in several studies in India. Rotavirus infections are responsible for 34% of all hospitalizations due to diarrhoea [7].The proportion of severe diarrhea attributable to rotavirus has increased from an average of 25% in studies which were completed prior to 2000 to over 38% in studies that were completed after 2005. A similar increase has been observed worldwide. Improvements in sanitation and use of antimicrobials would have had a greater impact on preventing bacterial and parasitic gastroenteritis than rotavirus gastroenteritis.[3]

Representative data on prevalence of rotavirus from all parts of the country is necessary to estimate the disease burden, prevalent strains of rotavirus in India and the expected health benefits of rotavirus vaccination.

1.2 History

Despite the magnitude of the problem of infantile diarrhoea the search for etiologic agents were unrewarding until the 1970s. [4] Infectious causes of diarrheal disease in children was detected in less than one third of cases till the early 1970s.[8] The important role of viruses in diarrhea began to emerge in 1972 with the discovery of

27nm Norwalk virus detected in an outbreak of viral gastroenteritis by Kapikian *et al* .[4]

This was followed by the discovery of 70nm human Rotavirus in 1973 by Bishop *et al* who observed a virus particle in the intestinal tissue of children with diarrhoea by electron microscopy establishing the association of this virus with diarrhea in infants and young children. [9] As the newly discovered virus resembled a wheel ,it was named as Rotavirus.USA declared that rotavirus was the organism responsible for diarrhoeal diseases among under five children in the year 1980.

Though discovered recently ,rotavirus contributed a major cause of gastroenteritis in both industrialized and resource poor countries. It soon became apparent that about 35%-50% of the hospitalizations for diarrhoeal disease upto two years of age were due to rotavirus . Rotavirus positive cases were reported from many parts of the world in paediatric patients with diarrhoea. Rotaviruses were the long-sought viral agent consistently outranking in importance than any other known causes of severe diarrhoea.

1.3 Virion Structure

Rotavirus particles have distinct morphologic appearance, and three types of particles can be observed by EM . The complete particles resembled a wheel like structure with a smooth outer surface without any

projections. The name rotavirus was coined based on this morphology . The complete infectious particles (Virions) are also called as triple-layered particles (TLPs). Double-layered particles (DLPs) lacking the outer shell are described as rough particles because of the presence of projecting trimeric subunits of the inner capsid. Single –layered particles (SLPs or cores) are seen infrequently which usually lack genomic RNA and are aggregated.[4]

1.4 Genome Structure and Organization

The viral genome consists of 11 segments of dsRNA, contained within the virus core capsid . The virion includes six structural viral proteins VP1, VP2, VP3, VP4, VP6 and VP7 and six nonstructural proteins (NSPs) NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6. As the virus possess segmented genome, the virus undergoes genome rearrangements resulting in new reassorted viral strains. Replication occurs in the cytoplasm and the viral particles are released by a Golgi independent vesicular transport which does not result in major cytopathic effects.

1.5 Classification

Rotaviruses are classified serologically into groups (serogroups) and multiple serotypes within each group. Rotaviruses comprise seven distinct groups (A to G). The group antigenic determinants are found on

most of the structural proteins and on many of the nonstructural proteins also. Group A, B, and C rotaviruses are found in both humans and animals, while groups D,E,F, and G rotaviruses have been found only in animals till date .

Group A rotaviruses have been established as a significant cause of diarrhoeal disease in infants and children and also in the young of several mammalian and avian species. Rotavirus B causes outbreaks of gastroenteritis occurring especially in adults. Group C viruses have been detected sporadically in children with diarrhoea and in several family outbreaks.

Rotaviruses have the ability to undergo reassortment of genes, but reassortment does not occur among viruses of different groups. Viruses with rearranged genome segments can replace the normal RNA since they are not defective.Genome rearrangements may play a role in evolution of rotaviruses in addition to antigenic drift and shift.

Rotaviruses are classified by a binary system similar to that used for influenza viruses. According to the genetic and antigenic diversity of two outer capsid proteins, VP4 and VP7 they are classified into several P types and G types.The fourth segment of gene codes for subtype P which is susceptible to protein cleaving enzymes encodes VP4. Seven,eight and ninth segment of gene code for subtype G which encodes VP7. Around

15 G types and 20 P types are recognized. The two genes that code for VP4 and VP7 have the ability to separate and unite individually forming recombinant G and P types. Epidemiological studies have proved the occurrence of genotypically mixed infections These proteins are immunogenic and induce neutralizing antibodies.Viral protein subtyping is important for the surveillance of rotavirus subtypes circulating in communities to develop vaccines for rotavirus.

Rotavirus Diversity

Rotaviruses display a great degree of diversity. Within group A, rotaviruses are classified into subgroups I, II, I+II and nonI, nonII depending on the reactivities of antibodies to epitopes on VP6 .There are different G and P types and a variety of combinations of these types also exist. Rotaviruses also exhibit intratypic variation. The co existence of recombinant rotavirus and their mutants in an area,warrants integrated monitoring of all prevalent strains.

1.6 Pathogenesis and Pathology

Pathogenesis of enteric infection is multifactorial involving both host and viral factors.

Multiplication of rotaviruses occur in the uppermost part of the villus in the differentiated intestinal cells.The mature intestinal cells may

have the factors necessary for the attachment and multiplication of rotavirus.

Several rotaviral proteins function as virulence factors which includes both viral protein and nonstructural proteins.These proteins may influence the multiplication of rotavirus.(VP3, NSP2, VP6, NSP3), turn off host protein synthesis (NSP3), entry into cells (VP4 and VP7), spread of infection beyond gut (NSP3 and VP6), regulation of the induction of interferon (NSPI), and the induction of diarrhea (NSP4).

Malabsorption secondary to destruction of enterocytes, ischemia of intestinal villi, an enterotoxin (NSP4), and activation of the enteric nervous system are the different mechanisms attributed to diarrhea caused by rotavirus infection.

Due to the impaired function of intestinal villi, normal transport of sodium,glucose and water is affected.This along with reduced amount of carbohydrate hydrolyzing enzymes result in osmotic watery stools.

1.7 Resistance

Rotaviruses are thermostble and not affected by temperature, light and changes in pH. Rotaviruses are found to be stable at ambient temperatures and in the low infectious dose for humans . Rotaviruses are also found to survive on various surfaces under different conditions contributing to the rapid spread of these agents.The large number of viral

particles shed in feces and relative resistance to physical inactivation may be responsible for infectious spread of disease causing virus. Effective disinfection of contaminated material and careful handwashing are the important measures to prevent rotavirus infection, especially in a hospital setting .

1.8 Clinical Features

The signs and symptoms of infection caused by rotvirus resembles other diarrheal diseases and does not have any distinguishing feature. The incubation period of infection has been estimated to be less than 48 hours.Children between 6 months to 2 years are most frequently affected. Next highest frequency of the illness is seen within 180 days of birth .Malnutritionis a key factor in increasing the severity of clinical manifestations rotavirus. Rotavirus causes mild to severe gastroenteritis characterised by watery diarrhoea, vomiting, and low-grade fever. The illness usually lasts for 3-5 days, but virus shedding may continue for about 10 days to 1 month. [10] Nosocomial infections with rotavirus are also reported frequently .

Complications

Infants and young children may suffer from severe diarrhea, dehydration, electrolyte imbalance and metabolic acidosis due to Rotavirus infection.Isotonic diarrhea of rotavirus infection causes severe

water loss compared to other diarrheal causative agents and is the reason for mortality due to rotavirus.Immunocompromised children may experience severe or prolonged rotavirus gastroenteritis resulting in abnormalities of multiple organ systems, particularly in liver and kidney.

1.9 Epidemiology

- Most common cause of diarrhea reported throughout the world
- Vulnerable age group of rotavirus infection is 6 months to 2 years
- No discrepancy of rotaviral diarrheal disease exist between industrialized and resource poor countries. Therefore the incidence of rotavirus infection cannot be altered by improving water or food sanitation.[11]
- Primary rotavirus infection usually does not give rise to permanent immunity. However subsequent incidences of diarrhea are of reduced severity may be due to partial acquired immunity

1.9.1 Reservoir

Infected human, harbouring rotavirus in the gastrointestinal tract and stool are the main reservoir of infection. Existance of true carrier state in rotavirus infection is yet to be proved though prolonged shedding is seen in immunodeficient persons. Rotavirus infection also occurs in many nonhuman mammals.

A role of animals as a source of rotavirus infection of humans has been suggested. Some animal rotaviruses share a common antigen with human rotaviruses and some naturally occurring animal rotavirus may

infect humans or undergo genetic reassortment with human rotaviruses. Human-bovine reasortant strains or a human-porcine reassortant strain (G5) have been endemic in selected areas in India or Brazil respectively.Human-simian rotavirus ressortants have also been detected in India .

1.9.2 Transmission

Rotaviruses are excreted in more numbers in the stool of affected individual. One gram of stool of an infected person may have around 10 trillion infectious virus and less than hundred particles are needed for transmission of infection from preson to person. The duration of rotavirus excretion in hospitalized children was in a range of 4 to 29 days, with a median of 7 days measured by enzyme immune assay.Detection by a more sensitive PCR assay may reveal shedding of rotavirus from 4 to 57 days, with a median of 10 days

Transmission is through feco-oral route, both by either by interpersonal contact or by inanimate objects. Food and water polluted with feces may cause the disease.Aerosol transmission has also been postulated.

1.10 Diagnosis

It is clinically not possible to differentiate the diarrhea caused by rotavirus and other infections.Therefore, an accurate diagnostic assay is necessary to establish rotavirus as a causative agent of gastroenteritis because children with rotavirus infectios need not be given any antibiotic except for other co existing bacterial infections.Inappropriate use of antibiotics enhances the emergence of resistance in bacteria and also adds the cost of treatment and risks of adverse reactions.

Fecal samples taken within 24 -96 hours of onset of diarrhea diarrhea are the best samples for diagnosis of rotavirus infection.Detection can be done upto 21 days if the illness is severe. The duration of diarrhea usually coincides with viral shedding but may extend even after the cessation of viral shedding

Several techniques have been developed to detect rotavirus in stool.Detection by electron microscopy is now practiced according to its availability. At present rotaviral disease is confirmedviral antigen detection in stool sample.Less time consuming ,easy to perform tests like immunochromatographic tests, latex agglutination and enzyme immunoassays are commercially available now.Reverse transcription polymerase chain reaction (RT-PCR) of faeces is used as a diagnostic

method in few laboratories to identify of specific rotavirus groups and types involved in outbreaks.

1.10.1 Electron Microscopy (EM)

Diagnosis rotavirusof rotavirus was initially done by EM . Approximately 10^6 viruses /ml of stool sample is required for detection by EM. This level of shedding occurs typically during the first 48 hours of illness. When tested at this period.The high accuracy of EM is due to the prescence of plenty of organism in the sample during the initial stages of infection and also the typical appearance of rotavirus. This is the rapid method when only a few specimens have to be examined.

All recombinant forms of rotavirus and its mutants can be visualized by EM,which are usually missed by the routine laboratory tests using the viral proteins.

The usage of EM is restricted,because it is expensive,unavailable in all centres and time consuming in examining large number of specimens.

Compared to conventional antigen detection tests and PCR, sensitivity of EM is low. Specificity of EM is comparable to Enzyme immunoassay, Latex agglutination. For grouping or serotyping the virions immune EM is preferred.

1.10.2 Culture

Viral culture is not done routinely to diagnose rotavirus infection.As the method is time consuming, technically demanding, and expensive this is not used as a routine diagnostic tool .The efficiency of this technique is lower to that of rapid antigen detection tests.

1.10.3 Antigen-detection methods

Most commonly used tests detect the presence of viral proteins of GroupA rotaviruses.

a) Enzyme immunoassay (EIA)

EIA is the frequently used specific diagnostic tool of rotavirus.The advantages of this method are that it does not require special instruments, has a built in control and many samples can be processed at a time. This is a sensitive method of detection and WHO has adopted this as the standard diagnostic technique for epidemiological studies done in developing countries like India. EIAs are also available to detect group B or group C rotaviruses. They are designed to detect antigenemia in blood samples of children with rotavirus diarrhea.

b) Latex Agglutination

A cheap and easily performed method to detect rotavirus A. Ability to detect rotavirus is similar to EIA,higher than EM and lesser than PCR.[12]Negative results of samples taken late in the illness or in a rectal swab should be confirmed by another method.Advantages of this

test are its easy interpretation and it is a rapid test. The test is suitable for routine use in small hospitals, emergency wards and in field studies for rapid diagnosis of rotavirus gastroenteritis.

c) Immunochromatographic tests

These simple to use, "point-of-care" immunochromatographic tests are widely used to detect rotavirus gastroenteritis in laboratories. Test is based on the principle of sandwich immunochromatography using two types of antibody in a solid phase to detect group A specific proteins.

Comparison of the efficiency of various diagnostic tools , an Enzyme Immuno Assay kit, latex agglutination kit and immune chromato graphic kit , keeping RT-PCR as a gold standard test revealed that sensitivities of these tests were 96%, 68% and 99% respectively.[13]

1.10.4 Antibody detection

Serology is not used as a diagnostic tool since high seropositivity is observed in the majority of older children for group A rotavirus. These tests can be used for epidemiologic studies of rotavirus.Rotavirus IgM is identified in blood 7 days after the commencement of illness but stool is a better specimen available to be tested at the time of presentation. Coproconversion of stool examined on the first day and seven days after the infection is ideal marker of reinfection.

1.10.5 Molecular methods

Reverse transcriptase polymerase chain reaction (RT-PCR) is the most sensitive molecular method available to detect rotavirus when done with the appropriate choice of primers. It can be used to detect nonA rotavirus infections also. Using Primers, representating all the types of rotavirus in a particular area will increase the detection rate. RT-PCR is 100-1000 times more efficient than conventional PCR.[14] Subtyping of rotaviruses are performed using specific primers against VP7of rotavirus.

1.10.6 Subtyping Methods

The frequently used test for typing of rotavirus is by enzyme immune assays which use specific antibodies to viral protein 7.A multiplexed heminested polymerase chain reaction is now available for genotyping based on VP7 sequences.

1.10.7 Choice of the test:

The performance of rapid tests indicated that these tests can only be used for screening but not for confirmation of infection. Therefore, a test with high specificity and sensitivity is mostly needed for confirmation of the rotavirus infection.Further subtyping is also essential to know the prevalence of subtypes of rotavirus in an area since vaccine has to be made specific to prevent that particular subtype.

1.11 Treatment

There is no specific antiviral therapy for rotavirus infection. Rehydration remains to be the mainstay of treatment since diarrhoea due to rotavirus leads to severe dehydration than most of the other causes.The recommended treatment is oral rehydration solution,proper intake of food and additional supplementation of zinc,similar to the management of diarrhoea due to other infectious causes.

1.12 Prevention

RotaTeq (RV5) and Rotarix (RV1) are the currently recommended vaccines. RotaTeq is a live oral vaccine containing five reassortant rotaviruses from human and bovine rotavirus strains. RV1 (Rotarix) is also a live oral vaccine containing one strain of live attenuated human strain of rotavirus. Efficacy of the vaccines in prevention of severe gastroenteritis by rotavirus is proved by several studies. RotaShield ,an older rotavirus vaccine was associated with an increased incidence of intussusception and is no longer used.

Breast feeding has protective effect against rotaviral diarrhoea as for diarrhea caused by any other pathogen.

Hand hygiene also plays a role in containment of rotavirus infection especially in hospital settings to prevent nosocomial infections.

Sentinel surveillance for severe rotavirus gastroenteritis is recommended by WHO to monitor vaccine impact. Currently, there is a network of sentinel hospitals which test the young children with diarrhoea for rotavirus infections and reports are submitted to WHO.[15] 10 hospitals and 4 laboratories throughout India are integrated in IRSSN(Indian Rotavirus Strain Surveillance Network). The diarrhoeal cases in under five age group who requires in patient observation and fluid replacement for a minimum period of six hours are registered and investigated for rotavirus .

Rotavirus continues to be the leading cause of diarrhoea in children less than two years of age, though a vaccine is available. The epidemiology and burden of rotavirus diarrhoea is fairly well documented in India. Adequate surveillance and introduction of rotavirus vaccine into the Universal immunization programme are the important part of efforts to reduce diarrhoea mortality which is the third leading cause of death among Indian children.[5] Rotavirus infections are frequently underestimated since routine laboratory diagnosis is not done. This study conducted in and around Tirunelveli aims at detecting rotavirus in stool samples of children with diarrhoea by rapid immunochromatography card test and to compare the efficiency of rapid card test against RT-PCR, a gold standard test in detection of rotavirus in fecal samples.

2. AIM AND OBJECTIVES

- To find out the prevalence of rotavirus associated diarrhoea in children less than 2 years.
- To evaluate the rapid immunochromatographic test in rotavirus antigen detection.
- To compare the efficiency of rapid ICT against RT-PCR in detection of rotavirus infection

3. REVIEW OF LITERATURE

Diarrhoea is the single most important reason for illness and death in developing nations. According to the World Health Organization and UNICEF, there are about two billion cases of diarrhoeal diseases occur worldwide annually, and 19 lakhs children under the age of five years are in developing countries.[16] Viruses are the commonest cause for diarrhoea in children , most common being rotavirus. As viral etiological agents are not investigated in all diarrhoeal cases, the exact number of viral diarrhoeal cases in the population is not available. By following proper investigating protocols for diarrhoeal disease ,unwanted use of antimicrobial agents can be avoided which will reduce the economic burden of treatment. Systematic epidemiological data collections regarding the total number of viral diarrhoea cases is very important in implementation of prevention programmes like vaccination in developing countries.

3.1 Burden of Diarrhoeal Diseases

A report by World Health Statistics 2011 says that death rate in children less than five years has reduced by thirty five percentage over a period of ten years throughout the world .Still, twenty one thousand deaths occur daily due to preventable reasons. Death toll under the age of five years persistently remains in large numbers in Africa and South-

east Asia which contribute 75% of the global burden. About 50% of the mortality occur in the following nations, such as China ,Democratic Republic of the Congo, Ethiopia, India, Nigeria, and Pakistan. Preventable diseases which include diseases of newborn, respiratory illness, diarrhoeal diseases, malaria and measles are responsible for 75% of childhood mortality. The above mortalities can be prevented by simple, cost effective interventions. [17]

3.2 Prevalence of rotavirus disease

With the use of sensitive molecular techniques, it is been found that most of the childhood diarrhoea are due to enteric viruses. Rotaviruses are the leading cause of diarrhea in children, followed by noroviruses in most of the countries. There is increasing evidence that both rotaviruses and caliciviruses may cause extraintestinal infections. [18]

3.2.1 Global Prevalence

The morbidity and mortality in children by rotavirus infection was analysed by CDC based on studies published from 1986 to 2000. Each year rotavirus causes around 111 million episodes of gastroenteritis requiring only home care, 25 million clinic visits, 2 million requiring hospitalizations, and 352,000–592,000 deaths in children less than five years of age. By the age of 5 years , nearly every child will have suffered from an episode of rotavirus gastroenteritis, 1 in 5 children will visit a

clinic, 1 in 60 will be hospitalized, and around 1 in 293 will die. Children in the poorest countries account for 82% of rotavirus deaths. [19]

The occurrence of rotavirus illness was uniform worldwide. Mortality is more in resource poor countries, due to difficulty in approaching treatment centres and an increased number of children with nutritional disorders.

S. M. Cook *et al* [1990] compiled results of various studies regarding the causes of gastroenteritis in children which showed 11-71% of illness was caused by Rotavirus. [20]

Rotaviruses are the most common cause of severe diarrhoeal disease of infants and young children globally , responsible for approximately 527,000 deaths per year. More than 85% of these deaths occur in low-income nations in Africa and Asia. Around two million children are hospitalized each year with severe dehydration due to rotavirus infection. In 2009, the Global Surveillance Network for rotavirus reported that, out of 36% of hospital admissions for childhood gastroenteritis, most susceptible age group was between 6 months and two years. [15]

In a study at Italy Rotavirus infection was detected in 27.6% of children aged 0 to 13 years over a period of 2 years, from 2005 to 2007 using a rapid immunochromatographic assay.[21]

A retrospective study in Istanbul, by Akan H *et al* during 2007-2008 detected Rotavirus in 18.7% patients with diarrhoea and/or vomiting, by a qualitative immunochromotographic assay.[22]

In a study conducted at Ryadh over a period of two years from 2008 in childhood diarrhoea cases revealed 65.5% was caused by rotavirus.[23]

Surajudeen A *et al* at Nigeria in 2011 reported 13.8% of rotavirus in stool samples of children with acute diarrhoea by enzyme linked immunosorbent assay .[24]

3.2.2 Prevalence in India

The contribution of rotavirus in childhood morbidity and mortality in developing countries like India is significant and reports from various parts of India documents the burden of rotavirus infection in children less than two years of age.

A study conducted at Tirupathi by Anand *et al* in 1992 reported rota virus infection to be 23.5% among children tested by ELISA.[25]

Rotavirus was detected in 28.15% of faecal specimens collected between 1992 and 1996 from children less than 5 yr of age in a study conducted by Kelkar SD *et al* in NIV Pune.[26]

A nationally representative survey done for three years from 2001 by Shaun K Morris *et al* revealed that the overall rotavirus-associated

mortality rate was 4.14 per 1000 live births. The mortality rate was 4.89 among girls and 3.45 among boys. Highest rates were reported in Bihar, UP and MP, contributing more than 50% of deaths occurring nationally.[27]

A cohort study results of fecal samples of children examined by electron microscopy during the year 2011 showed an overall rotavirus positivity rate of 37.9 % in Mumbai.[28]

3.2.3 Prevalence in Tamilnadu

A study conducted by P.K. Rajesh *et al* in 2005 among children under five years of age in Chennai, detected rotavirus antigen by latex agglutination in 27.45% and in 37.25% of children by Enzyme Immunoassay.[29]

Gagandeep Kang *et al* at vellore conducted a study of childhood diarrhea cases as per the recommended protocol of the Indian Rotavirus Strain Surveillance Network which has been established with 4 laboratories and 10 hospitals in 7 different regions of India. Rotavirus was found in approximately 39% of 4243 enrolled patients from 2005 to 2007.[30]

Retrospective analysis by Indrani Banerjee *et al* at Vellore in 2006 detected 7.1% rotavirus diarrhea in the community where as 27.4% in the hospital.[31]

A recent study by Kahn *et al* in 2012, says that rotavirus accounts for approximately 40% of hospitalizations for diarrhoea in India, with recent studies showing an increased proportion compared to older studies. The proportion of severe diarrhoea attributable to rotavirus infection has increased from an average of 25% in studies completed prior to 2000 to over 38% in studies completed after 2005.[32]

3.2.4 Prevalence of Rotavirus types in India

Rotaviruses isolated in India express a high degree of genetic diversity. The most frequently isolated strains from the children with diarrhoea are G1P[8] and G2P[4] .

In 2003 a study conducted at AIMS by Shobha Broore *et al* reported the most common types from India were Gtype1 , Gtype 2 and Ptype4 , Ptype 8. G9 strains were also commonly reported in Indian children. P6 strains of probable bovine origin have also been reported from India. In addition a novel neonatal strain P type 11 human rotavirus (116 E) was isolated from neonates in Delhi. The VP4 of this novel strain was closely related to the bovine serotype G10P[11] and VP7 was closely related to the human serotype G9. Similar neonatal strain G10P[11] was also reported from Bangalore. The bovine G10P[11] strains have a high prevalence in calves with diarrhoea.[33]

Saravanan P *et al* in 2004 has reported that the most common rotavirus strains isolated were rotavirus with short E-type, SGI and serotype G2 in 66.1% of the samples. The study also reported 10 different E-types and mixed genotype specificities with G2 P[4,8] and G1-G2 P[4,8] documenting the prevalence of genetically diverse rotaviruses in this area.[34]

Analysis of data from 18 epidemiological studies of Indian cities during 1996–2001 was done by Gagandeep Kang *et al* in 2011 to collect information on serotyping and genotyping of rotaviruses.Marked geographic differences in virus circulation was observed in India. G1 was the single most common G type reported in all parts of India, except for western India. Group B rotaviruses were reported from studies conducted in Kolkata and Pune.[35]

A recent study by Kahn *et al* in 2012, says that there is substantial evidence of serotype diversity in India, than previously thought. G1P[8] and G2P[4] are the two genotypes account for around 50% of symptomatic infections in non-neonates.[32]

3.3 Risk factors predisposing to rotaviral infection

3.3.1 Age factors

Severe rotavirus gastroenteritis requiring hospitalization characteristically occurs in more frequency in infants and young children

from 6 months to 2 years of age . The next highest frequency of such illness is experienced by infants under 6 months of age. In some studies, this age group had the highest frequency . [4]

Uhnoo and Svenson in 1986 observed that the age group with highest rate of rotaviral gastroenteritis was the 7-12 months old group.[36] Similar report was given by Lewis *et al* .[37]A study conducted at Tirupathi by Anand *et al* in 1992 also reported the highest incidence of infection among children in the age group of 7-12 months. [25]

A study done by Indrani Banerjee *et al* in 1992 at vellore reported the median age of affected children were lower (7.5 months) in the community than in the hospitalized children (10.5 months) with rotavirus diarrhoea.[31]

Analysis on prevalence of rotavirus diarrhoea among hospitalized children done by Kelkar SD *et al* from 1992 to 1996 at National Institute of Virology, Pune revealed that the age group of 6-24 months was the most susceptible one.[26] Similar reports were given by other studies conducted in India.[29,30]

A recent study by M Kargar *et al* in 2012 reported, children less than 24 months of age accounted for 70.83% of the overall rotavirus-positive cases with children between 9 and 11 months of age being the most affected .The median age was 7.9 months in the above study.[38]

Neonatal infections

Neonatal infections are often reported all over India. Most of the infections are asymptomatic. The role of neonatal infection protecting against subsequent infection remains unclear.

Sasirekha Ramani *et al* in 2006 reported 35 % neonatal rotavirus infections in a review of data from 46 epidemiological studies in India.[39] The strain that causes infections in newborn remains widely spread in the ward. Majority of the neonatal infections were asymptomatic and this may de due to passive immunity and incompletely developed intestine of the newborn.Neonatal rotavirus infections are expected to confer protection against subsequent infection and disease. Hence candidate vaccine strains can be derived from strains circulating in neonatal nurseries which includes two vaccine candidates currently under development in India. However, some neonatal infections were reported to be symptomatic, associated with devitalizing enteric infections, acute diarrhoeal diaeases and food intolerance.

Rotavirus antigens were detected in 16% of neonates born during the period from 2005 to 2006 at the AIIMS nursery in New Delhi, India.[40] Most of the rotavirus positive newborns in this study were between 5 and 9 days of age .

Rotaviral infection in adults

Rotavirus infection is usually asymptomatic, may occasionally present with diarrhoea .Adults are mostly involved in outbreaks. Three of 263 epidemic of acute diarrhoeal disease in the USA were found to be associated with rotavirus infection among adults.[41]

In a study conducted by Tatte V. S *et al* in NIV,Pune 16.2% of the adolescents 17.2% of the adults with acute gastroenteritis were found to be positive for rotavirus antigen.[42]

3.3.2 Gender

Varying reports are available about the incidence of rotaviral diarrhoea in male and female children. Although a male predominance was reported in some studies, the association was not consistent with other studies.

Analysis on prevalence of rotavirus diarrhoea among hospitalized children by Kelkar SD *et al* at National Institute of Virology, Pune, revealed that this disease was more predominant in males.[26] Similar male predominance in rotaviral infections was given by Trung Vu Nguyen *et al* [43] and by M Kargar *et al* .[38]

Where as a hospital-based study in children with rotavirus gastroenteritis by Sherchand JB *et al* found rotavirus detection was higher in female than male. But the difference was not statistically significant.[44]

3.3.3 Sociodemographic factors

Literacy, economic condition of the family and other sociodemographic factors do not have strong association with rotavirus infection.

In a study by V. Mishra *et al* ,no difference in incidence of infection was reported between children in rural and urban area in 2008 in India.[45] Similar results were given by a Turkish study. [46]

The results of a survey by K. L. Yap *et al* indicated higher prevalence of rotavirus associated gastroenteritis in children from lower socio-economic status. [47] The prevalence of the disease was higher in large families.Reduction in the risk of rotavirus diarrhoea was associated with hygienic home circumstances but not with the source of water supply used for drinking and washing .

No other sociodemographic or environmental factor except breast feeding had a correlation with rotavirus diarrhoea in a study in infants under 1 year of age, done by Abdollah B. Naficy *et al* .[48]

3.3.4 Season

Most of the rotaviral infections occur in winter season.However the seasonal effects are not clearly evident in tropical countries.

Analysis of various studies by S. M. Cook, *et al* reported that incidence of rotavirus infection was highest in winter in the Americas and

in the autumn or spring in the rest of the world. Specific seasonal patterns are not obvious in the tropics.[20] Persistence of rotavirus in the environment all through the year, suggest a possibility of low-level transmission maintaining the chain of infection.

A study in Washington D.C by C D Brandt *et al* in 1982 found eighty four percent increase in hospital admissions for rotaviral diarrhoea occured in cool,dry months than warm,wet ones.[49] This report is supported by a similar study done by Ahmed karadag *et al* in 2011.[50]

V. Mishra et al in northern India reported a rise in rotaviral diarrhoea in the middle of the year in addition to the cases that occur all through the year and also one clear cut rise during the winter.[45]The seasonal occurence of rotavirus infection is not uniform across the country due to the variation in the climatic conditions across the country. Various studies from India have identified a seasonal trend of the infection can be with two peaks of incidence in a year, or a single annual peak in winter or no specific pattern .

3.4 Transmission

Rotavirus is a highly communicable virus. Apart from the major faeco oral route, transmission via respiratory droplets is also a possible.Zoonotic transmission reulting in human infections is also

reported. A significant number of nosocomial gastroenteritis are due to rotavirus.

S A Ansari, *et al* reported that rotaviruses can survive on human hands for a variable period. [51]Around half of the inoculated infectious virus survived evan after an hour on human hands. In this evidence based study it was proved that rotaviruses could be transfered between animate and inanimate objects. These findings suggest that human hands play a potential vehicular role in transmitting the rotaviral infections.

Though the main mode of transmission of rotavirus is faeco-oral, spread by respiratory droplets has also been suggested. The increased occurence of infection in cooler months and its ubiquitous spread in all over the world is very similar to the viruses that spread by respiratory droplets, like measles.[20]

In a animal model study done by Prince DS *et al* ,rotavirus antigen was detected in the pulmonary and gastrointestinal tracts of the infected animals as early as 12 hours after the challenge with aerosolized rotavirus.[52] Antigen remained detectable in both sites for at least 8 days following infection leading to a clearly demonstrable gastrointestinal illness in the animals while there was no evidence of pulmonary pathology. These studies suggest that rotavirus infection can also be transmitted by aerosol droplets under experimental conditions.

Zoonotic transmission of bovine and porcine rotavirus strains G6, G8, G10, and G9P[19] were reported from human infections in western, southern, and eastern India by Gagandeep Kang *et al* in 2005.[53]

In India, 22.5% nosocomial enteric infections were reported by Sasirekha Ramani *et al* in 2008 from data of various epidemiological studies.[39]

3.5 Clinical features

The rotavirus gastroenteritis was remarkably consistent with sudden onset of vomiting, an increased frequency of fever and dehydration.[36]Respiratory symptoms may be present in some cases.

A study at Turkey ,Ahmet Karadag *et al* [2005] concluded that rotavirus-associated diarrhoea had a more severe clinical presentation than diarrhoea due to other causes.[50] More than half of all patients who required hospitalization were positive for rotavirus.

There are seven scoring parameters included in the Vesikari Clinical Severity Scoring System by Ruuska and Vesikari .[54] These parameters include the important symptoms in the clinical presentation such as diarrhoea, vomiting, fever, dehydration, duration of diarrhoea and vomiting and treatment status. Each of the seven parameters is equally divided into three according to the severity distribution except treatment status which was divided into two.The scores for each

parameter in the clinical severity scoring system are added to a severity score between 0 and 20 points.Scores more than 10 points are considered as severe, scores between 7 and 10 are moderate, and scores less than 7 are mild. If the scoring system is implemented correctly, approximately 50% of rotavirus positive cases should have a "severe" classification .

A study done at CMC Vellore by Indrani Banerji *et al* [2006] compared rotavirus diarrhea in hospitalized children and those in a community.[31] It reported that the mean severity of symptoms in terms of Vesikari scores were lower in the community.

3.6 Detection methods

Human rotavirus grow to very high titres in the human intestinal tract.It was calculated that one gram of faeces may contain a trillion of viruses.

Techniques for rotavirus detection includes Electron microscopy,Antigen detection methods including Latex agglutination, Enzyme immuno assays and Immunochromatographic tests and Molecular methods of Nucleic acid detection (PAGE), Nucleic acid amplification tests.

3.6.1 Electron microscopy

Peter J. Middleton, MD *et al* [1979] reported that electron microscopy discovered several viral etiologic agents from stool samples

of affected young children with acute gastroenteritis.[55] Rotavirus also known as reo-like agent emerged as an important etiological agent in viral gastroenteritis.During a period of one year, 669 patients were reported to be positive for rotavirus .Around one third of these infections were nosocomial.

A retrospective analysis of paediatric stool specimens by Nerurkar V *et al* 2011 was done using electron microscopy to analyse the prevalence of rotaviral disease in Mumbai revealed an overall rotavirus positivity rate of 37.9 %.[28]

3.6.2 Antigen detection methods

Latex agglutination tests

Sonia M raboni *et al* in their study concluded that the rapid,easy to perform,inexpensive latex agglutination test can be used to screen a large number of specimens though they give indeterminate results frequently.[12]

A total of 27.45% rotavirus antigen was detected by P.K. Rajesh *et al* in 2005 by Latex Agglutination in childhood diarrhea.[29]

co-agglutination test

Islam MN *et al* in 1995 developed and evaluated a co-agglutination test to detect rotavirus antigens in stool sample of patients with diarrhoea. [56] *Staphylococcus* cowan strain 1 coated with rabbit antisera against RV5 and SA11 rotavirus strains was used.This antibody coated

Staphylococci were specifically agglutinated by rotavirus present in stool samples within minutes.When evaluated against ELISA sensitivity of co-agglutination test was 76.19%, specificity 89.66% and positive predictive value and a negative predictive value were 57.97% and 95.26% respectively. The study suggests that the co-agglutination test is a simple and rapid test which can be used for screening of rotavirus infection in clinical practice.

ELISA

ELISA is the most commonly used method to detect rotavirus in many laboratories. This is the ideal screening test for rotaviral diarrhoea since it has high sensitivity.

Surajudeen A Junaid *et al* in 2011 detected rotavirus antigens in faeces of 13.8% children by enzyme linked immunosorbent assay technique.[24]Similarly Hamsa T Tayeb *et al* [23] kang *et al* [30] also used ELISA to diagnose rotaviral infection.

A study by Anand *et al* [1994] reported that ELISA had a sensitivity of 96.9% and specificity of 99.8%.[25]

ELISA for Antigenemia

Rotavirus Antigen in blood of Indian children with rotavirus diarrhoea and asymptomatic infections was detected by Sasirekha Ramani *et al* in 2010.[57] Children with rotavirus diarrhea had more

antigenemia than children with non-rotaviral diarrhea or asymptomatic infections .Antigenemia in rotavirus infection has a correlation with multiplication of the virus in the gut.

Immunochromatographic tests

ICT is used as a rapid screening test in diagnosis of rotavirus infection. This is suitable for diagnosing cases in a field level or in an outpatient department.

A study was done by Antonio Carraturo [2008] *et al* to detect the presence of rotaviruses and enteric adenoviruses in stool samples using an immunochromatographic assay. Rotavirus infection was detected in 27.6% cases.[21]

3.6.3 Molecular Methods

Nowadays the highly sensitive and specific molecular methods are used to diagnose rotavirus infections . These methods have the abilty to detect of infections with low viral load and are used as the gold standard technique in diagnosing rotaviral diarrhoea. Further strain typing is also done by this method.

Analysis by Nigel A. Cunliffe *et al* detected more than one virus in 53% of hospital acquired cases of acute gastroenteritis using PCR.[58] Among the viruses detected, rotavirus (31%) contributed one third while one fifths were due to norovirus and adenovirus 40/41.

Phillips G [2009] *et al* used ELISA to develop a cut-off in RT-PCR to attribute the illness to rotavirus in positive infectious intestinal disease cases and concluded that fixing a Ct cut-off value will help in interpretation of results of PCR. [59]

Petra F. G. Wolffs *et al* in 2011 reported that when the Ct values are high,its association with gastroenteritis is low.[60] A comprehensive PCR panel with appropriate cutoff values can be used as sensitive, rapid, and clinically relevant diagnostic tool for infection due to gastrointestinal viruses.

3.7 Comparison of detection methods

There are many methods by which rotavirus can be diagnosed. The choice of the test depends on the availability, cost, skill required and the efficiency of the test to detect the infection. A comparative analysis of the tests will help in selecting the method depending upon the situation.

A study conducted by P.K. Rajesh *et al* [2005] detected rotavirus antigen by latex agglutination in 27.45% of cases and in 37.25% of cases by enzyme immunoassay.[29] The study concluded that latex agglutination though a very simple and inexpensive bedside test failed to detect the antigen in 35.6% of the cases.

Regagnon C *et al* compared two rapid diagnostic tests , an enzyme immunoassay test (EIA) and an immunochromatographic test (ICG) in

detection of rotavirus antigen in stool samples.[61]Discrepant results of the specimens were resolved by electron microscopy (EM).Of the total samples tested, 30.3%were positive and 64.8% were negative by both tests. After confirming the discrepant results by EM the study concluded that an excellent concordance exists between the EIA and the ICG tests in spite of the underestimation of ICG test.

In a study Zeng SQ *et al* , compared the effectiveness of EIA against RT-PCR in diagnosis of rotaviral diarrhoea.[62] Q-PCR using specific primers and TaqMan probe against NSP3 was used . Q-PCR increased the detection rate by 28% by additionally detecting EIA negative samples.

A study by Kirsti Vainio *et al* [2009] compared the efficiency of ELISA, ICT and RT-PCR in the detection of rotavirus in stool samples.[63] The detection of rotavirus was 58% by the ICT, 63% by ELISA and 72% by RT-PCR. The study reported that specificity of ELISA was comparable to PCR while the sensitivity was low. Lowest sensitivity and specificity was reported for ICT.

Field evaluation of a rapid immunochromatographic test for rotavirus and adenovirus was done by Thomas Weitzel *et al* in 2006.[64]Results of the rapid test of stool specimens from children with acute gastroenteritis were evaluated against PCR. The sensitivities and

specificities of immunochromatographic test were 75% and 95% for rotavirus and 22% and 84% for adenovirus.The study suggested that ICTs may be used in diagnosis in resource poor settings.

3.8 Treatment

Rotavirus gastroenteritis is a self-limited illness, lasting for only a few days in persons with healthy immune status.No specific treatment is needed . Oral rehydration therapy to prevent dehydration is the mainstay in management. Around 1 in70 children with rotavirus gastroenteritis will require hospitalization for intravenous rehydration.[8]

The recommended World Health Organization (WHO) oral rehydration solution containing sodium, chloride, and electrolytes is the standard treatment for dehydration in anyone with acute gastroenteritis, including that caused by rotavirus .The rice-based oral rehydration solution is an easily metabolized carbohydrate formulation which helps to repair damaged tissues and enhances electrolyte absorption.[65]

Dubey AP *et al* [2008] New Delhi, India has reported in their study that use of probiotic mixture in acute rotavirus gastroenteritis resulted in decreased stool volume losses during diarrhoea leading to earlier recovery and decreased frequency of ORS administration.[66]

A clinical trial by Sarker *et al* [1988] reported that immunoglobulin from immunized bovine colostrums given to children

had significantly reduced the daily stool frequency and total stool output resulting in decreased requirement of oral rehydration solution than children who received placebo .[67]

3.9 Prevention

Practising Hand hygiene will prevent cross infection acquired within the hospital. Zerr DM *et al*[2005] found in his study that the incidence of hospital acquired rotavirus infection declined from around 6 episodes per one thousand patients in 2001 to around 2 episodes in 2004, after implementation of hand hygiene programme in a hospital.[68]

In a study Mastretta *et al* [2002] reported that rotavirus infection was less common in breast-fed infants than non-breast-fed infants.[69]

3.10 Vaccines

Vaccination against rotavirus was recommended by WHO in 2009, to be included in national immunization programs .

Two different types of rotavirus vaccines are currently recommended for use in infants in USA. The vaccines are RotaTeq (RV5) and Rotarix (RV1). Both vaccines were proved to be safe and effective.During the first year of an infant's life, rotavirus vaccine was found to prevent almost all (85%-98%) severe rotavirus illness episodes and to prevent 74%-87% of all episodes of rotavirus associated illness.[8]

The American Academy of Paediatrics recommended routine vaccination of infants with three doses of pentavalent rotavirus vaccine given orally at 2, 4, and 6 months of age. The first dose should be given to infants between 6 and 12 weeks of age.Infants older than 12 weeks of age should not be immunised. Subsequent doses are to be administered at four to ten week intervals, and all 3 doses of vaccine should be administered by 32 weeks of age. Pentavalent rotavirus vaccine can be administered with other vaccines of childhood. Infants who are highly sensitive to any constituent of vaccine is a contraindication of vaccine.A bovine based pentavalent vaccine (RotaTeq) for rotavirus was licensed on February 3, 2006, by the FDA for use in infants in the United States.[70]

Analysis by Manish M. Patel *et al* [2012] attempted to remove the age restrictions for rotavirus vaccination.[71] Given in an age restricted schedule vaccine would prevent 155,800 rotavirus deaths while causing 253 intussusception deaths in low and low-middle income countries.In contrast when vaccinated without age restrictions the same would prevent 203,000 rotavirus deaths while causing 547 intussusception deaths. Hence removing the age restrictions would lead to an increased benefit-risk ratio of 154 deaths prevented for every single death caused by vaccine. This study suggested that benefits of vaccination is more compared to the vaccine-associated intussusception deaths in low and

middle income countries and recommends removing the age restrictions for vaccination.

Nicaragua was the first developing country to introduce rotavirus vaccine into the vaccination schedule.[72] Studies in this country revealed that rotavirus vaccine prevented 60 % of severe rotavirus cases and reduces emergency clinic visits by half. In USA, rotavirus vaccine has reduced rotavirus associated hospital admissions upto 86 percent. The vaccines may also have a role in preventing the disease in non-vaccinated children by reducing the number of circulating infections.

3.10.1 Vaccine in India

Recommendations for Use

As the burden of morbidity and mortality due to rotavirus is acknowledged by IAPCOI ,inclusion of rotavirus vaccine in the regular vaccination schedule is recommended.[73]Due to the presence of genetically diverse rotavirus in Indian population, the effectiveness of the rotavirus vaccine will not be similar to that of in other parts of the world. Hence IAPCOI recommends rotavirus vaccination as an optional one.

Rotarix is an oral monovalent vaccine given in two doses at 6-12 weeks interval. Rota Teq is a pentavalent vaccine to be administered orally in three doses starting at 6-12 weeks of age. Serodiversity of

rotavirus in India favours a monovalent vaccine which can induce heterotypic immunity or a polyvalent vaccine that incorporates majority of serotypes prevalent in the nation. However, the efficacy of available rotavirus vaccines is less in developing countries like India.[74]

Rotavac (116E), an experimental rotavirus vaccine under development in India by Bharat Biotech company is in the final phase (phase III) of clinical trials.[75]

3.11 Surveillance

3.11.1Global surveillance network

The World Health Organisation coordinates an international surveillance programme by standardising the case definitions and diagnostic tests to detect rotavirus in paediatric patients at the level of sentinel hospital.[76] Data from various parts of the world is summarized by the network. In 2009 reports were obtained from 43 participating countries which have tested more than 100 stool samples for all twelve months in the year and rotavirus positivity was reported to be 36%.

Information on disease burden acquired from surveillance are important in making decisions about introduction of rotavirus vaccine into a country, and also to monitor the vaccine impact.

3.11.2 Indian Rotavirus Strain Surveillance Network (IRSSN)

Indian Rotavirus Strain Surveillance Network has been established with 10 hospitals in 7 cities such as in Vellore (Christian Medical College), New Delhi(Indian Council of Medical Research), Pune(National Institute of Virology) , Mumbai (Enterovirus Research Center and Lokmanya Tilak Municipal General Hospital), Chennai (National Institute of Epidemiology),Kolkata(National Institute for Cholera and Enteric Diseases) for sentinel surveillance.

As the morbidity due to rotavirus diarrhoeal disease is high in developing countries like India, surveillance regarding the prevalence of the illness and distribution of various strains in India is needed to intensify control measures.Proper management of acute diarrhoeal diseases, enhancement of vaccination programme in addition to promotion of breast feeding and practice of good hygienic procedures have to be integrated in the prevention programmes to achieve a significant reduction in the burden of the illness.

4. MATERIALS AND METHODS

The present study was conducted at the Department of Microbiology , Tirunelveli Medical College, Tirunelveli from May to November 2012 to detect the prevalence of rotavirus associated diarrhea in children less than 2 years of age and to evaluate the efficiency of rapid immunochromatographic test [ICT] in rotavirus antigen detection and also to compare its performance against Polymerase chain reaction [RT-PCR], the gold standard test.

4.1 Study group

A total of 100 stool samples were collected from eligible children.

4.1.1 Inclusion criteria

- Children less than 2 years of age with loose stool without blood and mucus.
- Children requiring hospitalization.

4.1.2 Exclusion criteria

- Children more than 2 years of age with loose stool.
- Children less than 2 years of age with loose stool with blood and mucus.

4.1.3 Ethical clearance

Ethical clearance was obtained from the college ethical committee before the commencement of the study.

4.1.4 Consent

Informed consent was obtained from reliable informants of all patients who participated in the study.

4.1.5 Questionnaire

Symptoms regarding the onset of diarrhoea, duration, presence of other clinical features like vomiting, fever and features suggestive of dehydration were recorded in a questionnaire.

History of rotavirus vaccination was also included in the questionnaire.

4.1.6 Study sample:

A total of 100 non duplicate stool samples were collected from the study group. Around 5 ml of sample collected in a sterile container with a spoon attached to the lid from the suspected cases in the acute phase of illness, preferably on the day of presentation to hospital. Attempts were made to collect the sample within 48 hours of hospital admission to avoid rotaviral infections acquired nosocomially.The specimen was properly labelled with serial number, name of the patient and date of collection.

4.1.7 Storage of sample

Samples were immediately tested for rotavirus by rapid immunochromatographic method and then stored in a buffer for RT-PCR at -80° C.

METHODS

All 100 samples were tested for rotavirus by both rapid immunochromatography and RT-PCR.

4.2 Immunochromatography

All the 100 samples were tested by rapid immunochromatography card test (SD BIOLINE Rota/Adeno kit).

4.2.1Test principle

Immunochromatography is a lateral flow immunoassay.It contains a chromatographic pad with three zones; sample pad, conjugate pad and capture line.The conjugate pad is impregnated with colloidal gold.The specimen when applied to the sample pad flows laterally by capillary action.Upon reaching the conjugate pad it binds to the antibody conjugate and foms antigen-antibody complex.This complex then flow laterally to reach the capture line where it is captured by the second antibody.The presence of coloured line indicates a positive result.

Rapid test cassette has a letter of “1” for Adenovirus and “2” for Rotavirus as Test line and “C” as Control Line on the surface of the device.

4.2.2. Materials provided

The rapid immunochromatographic test kit contains following items to perform the assay;

- BIOLINE Rota Rapid test device
- Sample collection tube
- Assay diluent
- Sample collection swab
- Disposable dropper

Active ingredients of main components

- 1 test strip includes ; Gold Conjugates (as main component) : colloidal gold conjugated mouse monoclonal anti-rotavirus antibody.
- TestLine1(as main component): Mouse monoclonal antiadenovirus antibody.
- Test Line 2(as main component) : Rabbit polyclonal antirotavirus antibody.
- Control Line (as main component) : Goat anti- mouse IgG.

- Assay diluent includes ; 20 mM Phosphate buffer , Sodium azide (0.01%.)

4.2.3.Kit Storage

- The Rapid test kit was stored at room temperature. (1 ~ 30° C)

4.2.4. Procedure of the test

Samples collected in sterile container without any additives and transport media was tested soon after collection.

- The assay diluent was taken up to the mark in the disposable dropper. And then, it was transfered into the sample collection tube.
- This step was repeated once again.
- A specific amount of sample (about 50mg) was taken with the sample collection swab.
- The swab was inserted into the sample collection tube with assay diluent.
- The swab was swirled at least 10 times until the sample has been dissolved into the assay diluent and the swab was discarded while squeezing the swab against the wall of tube.
- The dropping cap was assembled on the sample collection tube.
- 4~5 drops of the sample was added (about 100~125µl) into the sample well of the test device

RAPID ICT KIT

ROTA VIRUS POSITIVE

4.2.5. Interpretation of the test

Negative Result : The presence of only control band (C) within the result window indicated a negative result.

Adenovirus Positive Result : The presence of colored band ("1" band and "C" band) within the result window

Rotavirus Positive Result : The presence of colored band ("2" band and "C" band) within the result window.

Invalid Result : If the control band is not visible within the result window after performing the test, the result is considered invalid.

4.3.Real-Time PCR

All the 100 samples tested by rapid ICT were tested again with RT-PCR by the kit provided by Helini Biomolecules,Chennai,India and the procedure was done according to the manufacturer's guidelines.

4.3.1 Principle of Real time PCR

In Real-Time PCR the progress of amplification reaction is monitored by a camera in "real-time".A fluorescent marker which binds to the amplified DNA is used as a marker of progress.Measurement of products generated during each cycle of the PCR process is directly proportional to the amount of template prior to the start of PCR process. Every one copy of specific sequence is amplified and detected in an

exponential manner. As the number of gene copies increases, the fluorescence also increases which is easily detected.

The increase in the fluorescence emission in the PCR reaction can be detected in real time by a modified thermocycler.The computer software constructs amplification plots using the fluorescence emission data collected during the PCR cycle.

4.3.2 Safety precautions

All the laboratory works were carried out as per standard laboratory procedures and Bio-safety norms in Class II Biosafety cabinet

4.3.3 Instruments

- Vortex mixer
- Refrigrated centrifuge
- Thermocycler (Biorad CFX 96)
- Computer for data analysis and storage

4.3.4 RNA extraction

4.3.4.1 Components of RNA extraction kit

- Carrier RNA
- Lysis buffer
- Internal Control Template
- Wash Buffer-I
- Wash Buffer- II

- Ethyl alcohol
- Elution Buffer

4.3.4.2 Storage and stability

The kit can be stored at room temperature(15-25°C) for up to 12 months except carrier RNA which was stored at -20° C.

4.3.4.3 Sample preparation for RT-PCR

1-2 ml or 1-2 gm of fecal specimen from a patient with symptoms of gastroenteritis was mixed with a stool collection buffer provided by the manufacturer.

- sample was transferred to a 2ml centrifuge tube.
- 1.6ml stool buffer was added and vortexed well for 1minute.
- Then the sample was centrifuged at 14000rpm for 3 minutes.
- 1.25ml of supernatant was transfered into fresh 2ml tube.
- Clarified supernatant (~1-1.5 ml) was stored at 4-8° C for short term storage and –80° C for the longer term.

4.3.4.4 Principle of RNA extraction

The principle is based on the selective binding properties of RNA to a silica gel based membrane under centrifugation. The sample is first lysed under highly denaturing conditions to inactivate RNase and to ensure isolation of intact viral RNA. Carrier RNA added helps in optimum binding of sample RNA to the membrane .When the sample is

added to the spin column,the sample RNA binds to the membrane and contaminants are efficiently washed away in 2 steps using 2 different wash buffers. High quality RNA is eluted in a special RNase free elution buffer. The purified RNA is free of protein, nucleases and other contaminants and inhibitors.

4.3.4.5 RNA EXTRACTION PROCEDURE

1. 0.6ml of the prepared sample supernatant was transferred into fresh 2ml microcentrifuge tube.
2. 600 µl of lysis buffer and 6 µl of carrier RNA added and mixed well by vortex for 1 minute.
3. This mixture was incubated for 10 minutes.
4. Then 600 µl of ethanol [100%] was added and mixed well by vortex for 30 seconds.
5. 600 µl of sample was pipetted into the spin column & centrifuged for 1 minute.The flowthrough was discarded and the column placed back into the same collection tube.
6. Step 5 was repeated for the remaining processed sample.
7. 500 µl Wash buffer-I was added to the spin column.Centrifuged for 30-60 seconds and the flowthrough discarded .Then the column was placed back into the same collection tube.

8. 500 µl Wash buffer-I was added to the spin column.Centrifuged for 30-60 seconds and the flowthrough discarded . The column was placed back into the same collection tube.
9. Centrifuged for an additional 2 minutes and the column was then placed into a fresh 1.5ml microcentrifuge tube.
10. 60 µl of elution Buffer was added to the center of spin column membrane and the column was incubated for 1 minute.
11. The spin column was centrifuged for 1 minute and the column diacarded.
12. Centrifuged tube now contains eluted RNA.
13. Eluted RNA was either used immediately for RT-PCR or stored at -80°C for later analysis.

4.3.5 PCR amplification

4.3.5.1 Components of Rotavirus PCR kit

- RT Q PCR Master mix - contains the essential components for PCR amplification like Reverse Transcriptase enzyme, dNTP, Taq DNA polymerase,Taq reaction buffer, Mgcl2, and ribolock enzyme
- Rotavirus A Primer Probe Mix - The primer probe has a forward primer and a reverse primer specific for NSP3 region of rotavirus tagged with FAM as a fluorophore and BHQ1 as the quencher molecule.

- Forward Primer: ACCATCTWCACRTRACCCTC
- Reverse Primer: GGTCACATAACGCCCCTATA

- Internal Control Primer & Probe Mix to make sure that PCR inhibitors are not present in the extracted sample. Manufacturer recommends optional usage of internal control.
- Rotavirus positive template
- Nuclease free water

The kit was stored at -20° C

4.3.5.2 RT-PCR for detection of Rotavirus-A

In this study, real time Q-PCR was used to detect rotavirus in stool samples. Conversion of rotavirus RNA to cDNA copy by the enzyme reverse transcriptase and amplification of cDNA by Real-Time PCR to many copies takes place in one step.

Rotavirus A detection reaction mix

The reaction mixture is prepared by adding specific amount of the components provided by the manufacturer. A typical 25 µl reaction mix is prepared for the samples along with a positive control and a negative control.

Reaction mix for samples

5 µl of extracted sample is added to the reaction well along with 18 µl of Q PCR master mix and 2 µl of Rotavirus A primer probe mix in each well.

S.No	Components	Volume
1.	RT-QPCR Mix	18 µl
2.	Rotavirus A Primer Probe Mix	2 µl
3.	Purified RNA sample	5 µl
	Total volume	25 µl

Negative control & Positive control Reaction Setup

Negative control :

5 µl of negative control was added to the reaction in the place of purified RNA sample.

Positive control :

5 µl positive control was added to the reaction well in the place of purified RNA sample.

Note:

- Negative control was added first before any sample is added.

- Positive control was added last after sealing all samples and negative control.

Reaction type	Negative control	Positive control
RT-QPCR Mix	18µl	18 µl
Rotavirus A- Primer Probe Mix	2µl	2 µl
Purified viral RNA	XXX	XXX
Nuclease free water	5µl	XXX
Positive template	XXX	5 µl
Total volume	25µl	25 µl

The reactions were centrifuged briefly to remove the bubbles if any in the reaction mix since bubbles interfere with the fluorescence detection.Then the wells were placed in a thermocycler for amplification.

Amplification reactions were performed on a Biorad (CFX 96-Realtime system) Thermocycler with the following thermal conditions

Amplification profile

	Step	Time	Temp
	Reverse transcription	30min	50º C
	Taq enzyme activation	15min	95º C
40cycles	Denaturation	15sec	95º C
	Annealing/ Data collection	30 sec	55º C

- Results along with amplification curves were stored in the computer and computer print outs taken for further analysis.

4.3.5.3 Result interpretation

Ct value <37 with typical sigmoid shape amplification curve was taken as positive.

Ct value ≥37 are taken as negative.

Sample	Negative control	Positive control	Interpretation
Positive	Negative	Positive	Positive
Negative	Negative	Positive	Negative
Negative	Negative	Negative	Experiment fail
Positive	Positive	Positive	Experiment fail

BIOSAFETY CABINET

THERMOCYCLER

ROTA VIRUS RNA ISOLATION KIT

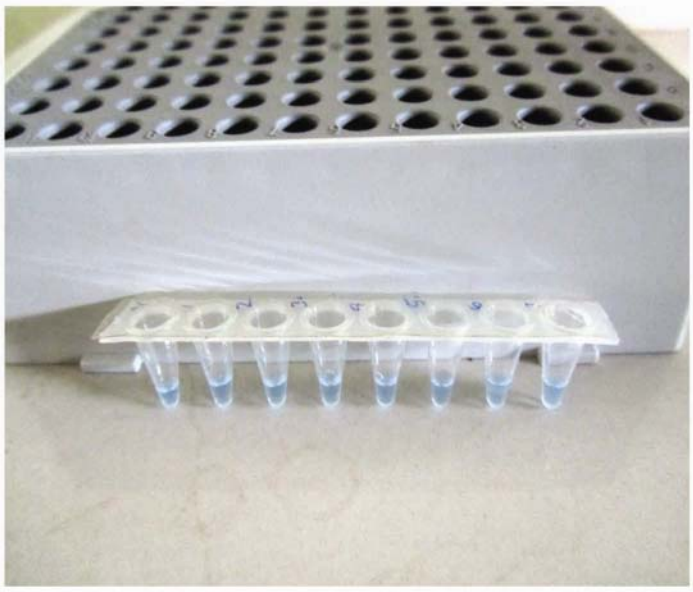
SPIN COLUMN SINGLE

EXTRACTED DNA

RT-PCR KIT

PCR REACTION MIX ARRANGEMENT

ROTAVIRUS REACTION MIX



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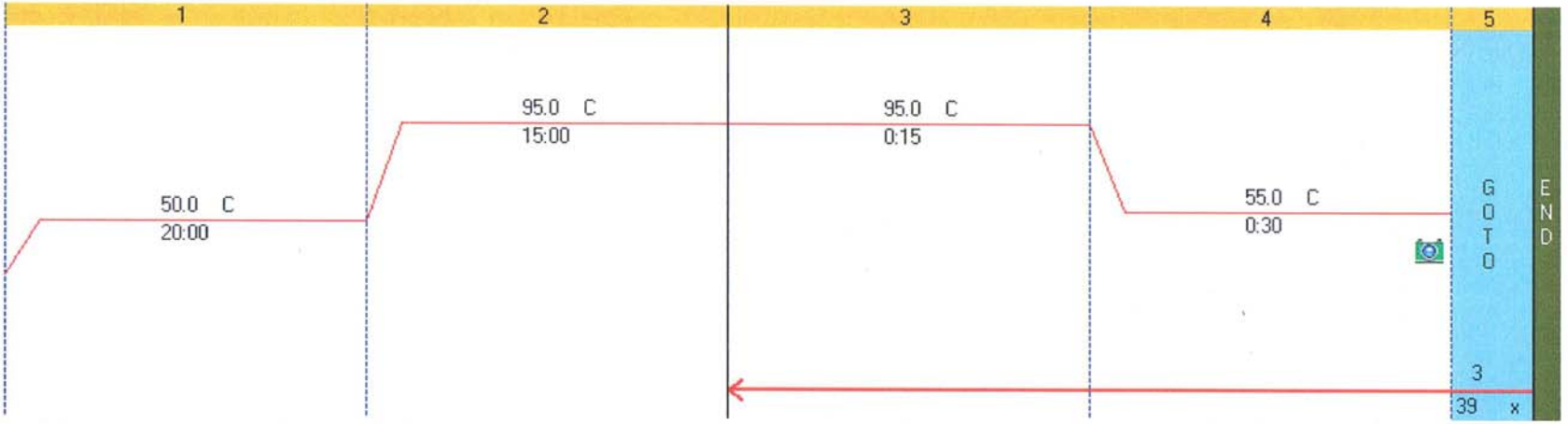


Insert Step

Insert Gradient

Insert GOTO

Insert Melt Curve

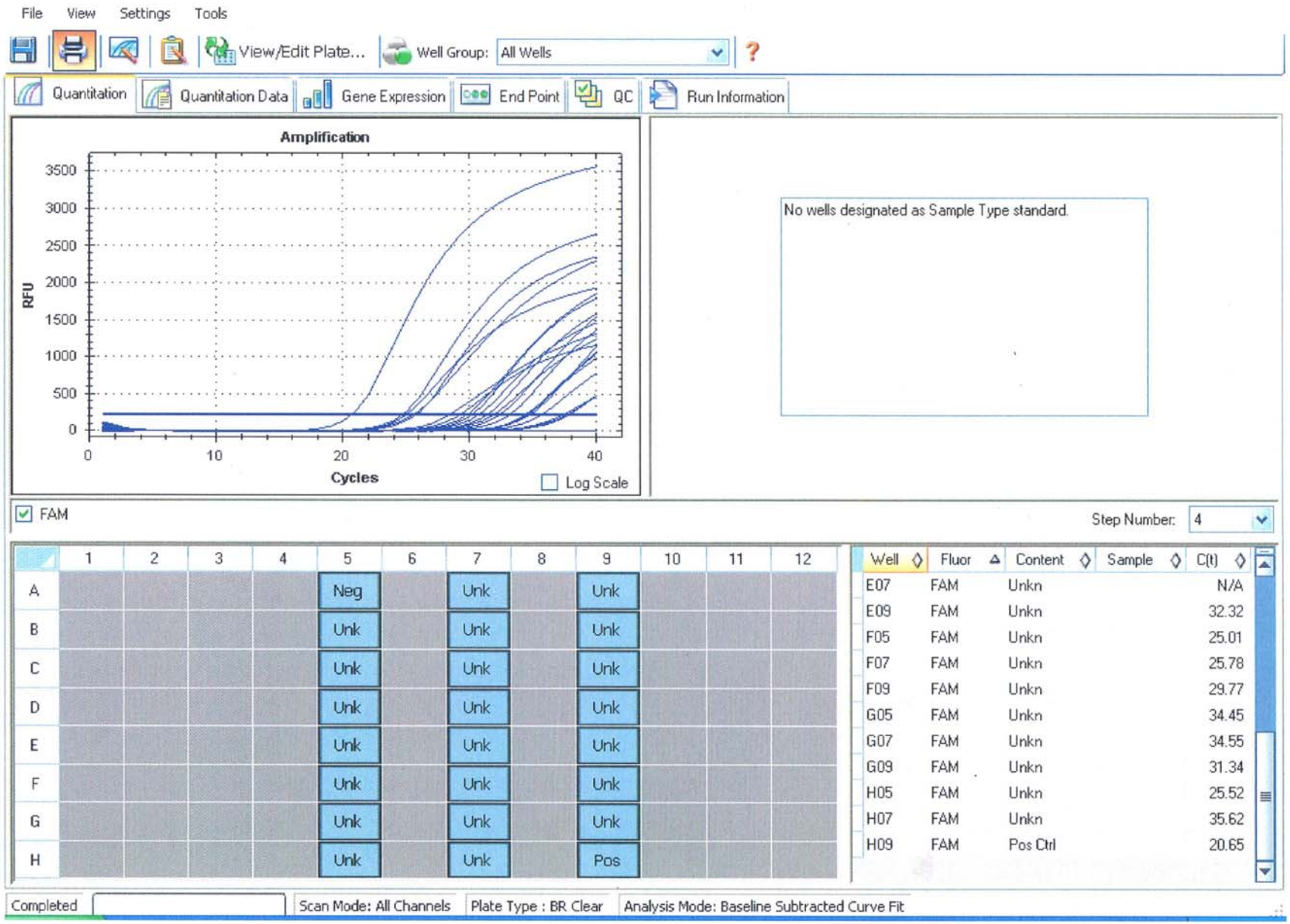
Add Plate Read to Step

Step Options

Delete Step

1 50.0 C for 20:00
2 95.0 C for 15:00
3 95.0 C for 0:15
4 55.0 C for 0:30
+ Plate Read
5 GOTO 3 , 39 more times
END

OK Cancel


File
View
Settings
Tools
View/Edit Plate...
Well Group:
All Wells
Quantitation
Quantitation Data
Gene Expression
End Point
QC
Run Information
Amplification
RFU
3500
3000
2500
2000
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1000
500
0
0
10
20
30
40
Cycles
Log Scale
No wells designated as Sample Type standard.
FAM
Step Number:
4
1 2 3 4 5 6 7 8 9 10 11 12
A Neg Unk Unk
B Unk Unk Unk
C Unk Unk Unk
D Unk Unk Unk
E Unk Unk Unk
F Unk Unk Unk
G Unk Unk Unk
H Unk Unk Pos
Well Fluor Content Sample C(t)
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E09 FAM Unkn 32.32
F05 FAM Unkn 25.01
F07 FAM Unkn 25.78
F09 FAM Unkn 29.77
G05 FAM Unkn 34.45
G07 FAM Unkn 34.55
G09 FAM Unkn 31.34
H05 FAM Unkn 25.52
H07 FAM Unkn 35.62
H09 FAM Pos Ctrl 20.65
Completed
Scan Mode: All Channels
Plate Type : BR Clear
Analysis Mode: Baseline Subtracted Curve Fit

5. RESULTS

5.1 The Study Group

A total of 100 children aged below 2 years who fulfilled the criteria of suspected cases of rotaviral gastroenteritis were analysed. This study was conducted at the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli over a period of one year from August 2011 to July 2012.

5.2 Statistical Analysis

All the results obtained from the study were analysed stasticially for their completeness, consistency and accuracy by the parameters like mean, median and percentages. The differences of above parameters were tested by the parametric tests like 'Z' and't' and non-parametric test like χ^2 test, which was applicable wherever. The results of Rapid ICT and RT-PCR were compared by Mcnemar's χ^2 test and confirmed by 'Z' test of proportions. The above statistical procedures were performed by IBM SPSS Statistics 20. The P- Values of less than 0.05 were considered as statistically significant in two tailed test ($P<0.05$).

5.2 Result analysis

The selected 100 study subjects were analysed based on age and sex. The results of the analysis are tabulated in Table 1.

Table 1. Age and sex distribution of study group

Age (months)	Male		Female		Total	
	No	%	No	%	No	%
00-06	14	27	9	18	23	23.0
07-12	29	57	27	55	56	56.0
13-18	8	16	10	20	18	18.0
19-24	0	0	3	6	3	3.0
Total	51		49		100	100.0

Of the 100 patients, 51 were males. Of this, 29 (57%) were in the age group of 7-12 months and 14 (27%) were less than 6 months of age. The remaining 49 were female children .Of this 27 (55%) were in the age group of 7-12 months and 10(20%) were in the age group of 13-18 months.(Fig 1)

The analysis shows that the mean age of male children with diarrhoea was 8.7 months and that of female children was 10.3 months.

Table 2. Distribution of associated clinical features in study group

Clinical features	Number of patients (n=100)	Percentage
Vomiting	69	69
Fever	62	62
URI	15	15

The above table (Fig2) shows that among the 100 cases studied,69% of patients had associated vomiting while 62% had fever and 15% had upper respiratory tract infection.

Fig.1 Age and sex distribution of study group

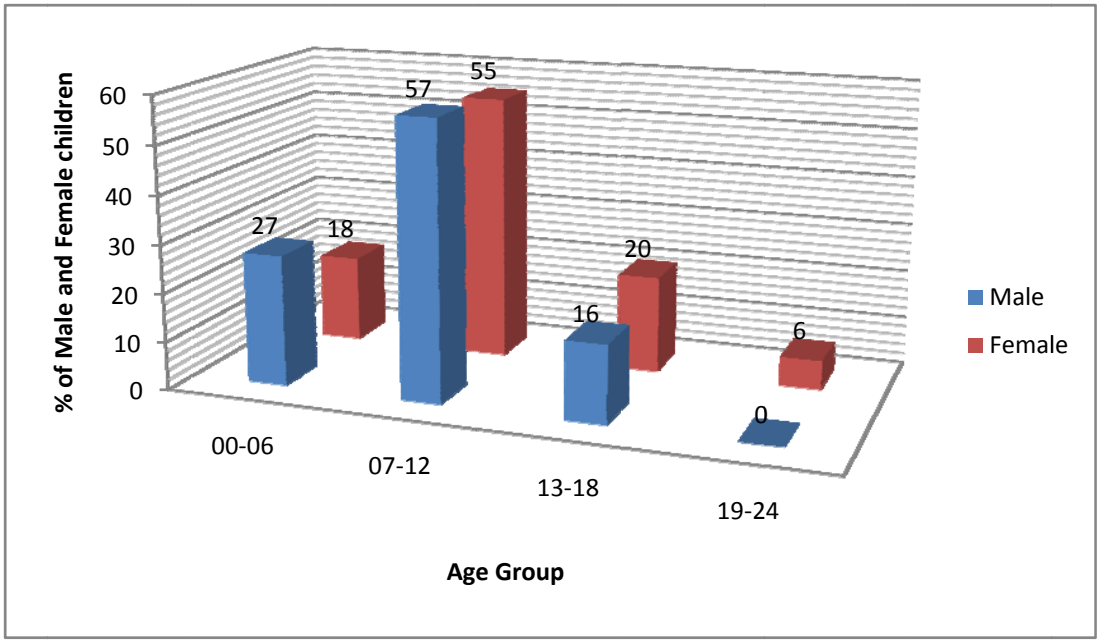


Fig 2 Distribution of associated clinical features in study group

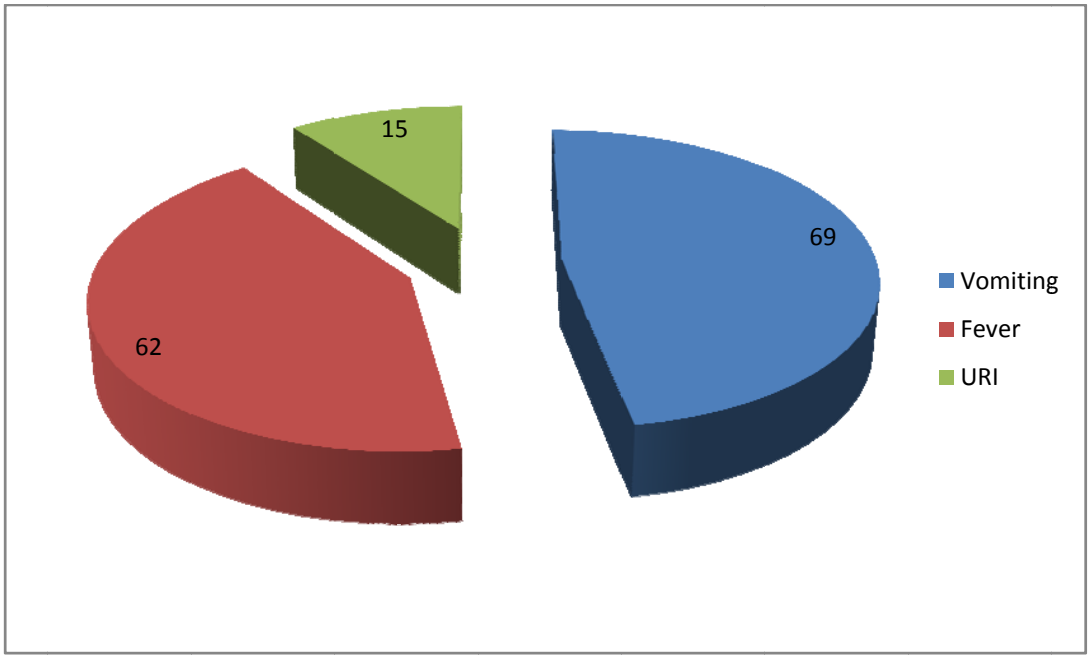


Table 3 . Comparison of rapid ICT and RT-PCR in detection of Rotavirus

Test	Samples tested	Positive		Negative	
		Cases	%	Cases	%
RT-PCR	100	64	64	36	36
ICT		34	34	66	66

All the 100 samples tested by both rapid ICT and RT-PCR for rotavirus in their stool samples.Out of this RT-PCR was positive for 64% of samples while 34% were positive by ICT as shown in table3.(Fig3)

Fig 3 . Comparison of rapid ICT and RT-PCR in detection of Rotavirus

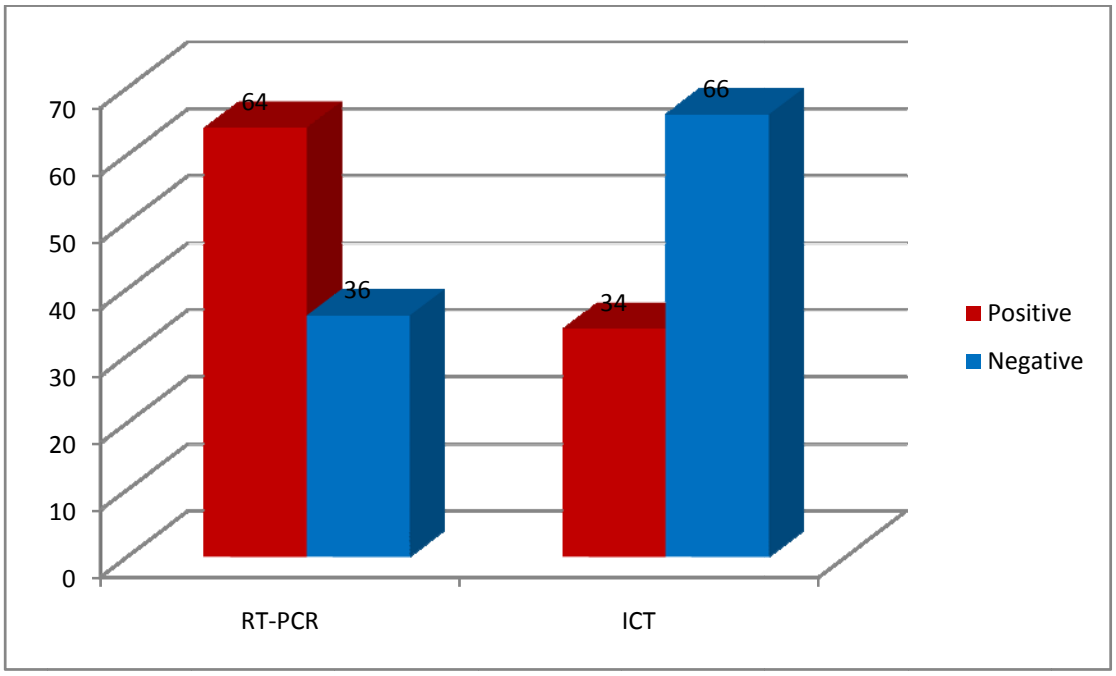


Table 4 Ct values of samples tested by RT-PCR

Ct value	Number of cases	Percentage
<20	0	0
>20 and < 30	29	29
>30 and < 37	35	35
≥ 37 and not amplified	36	36
Total	100	100

The distribution of Ct values of all the samples tested are depicted in the above table.(Fig 4).Out of all 100 samples tested by RT-PCR,29 samples had Ct values of more than 20 and less than 30. Ct value between 30 and 37 was observed in 35 samples.Among the 100 samples 11 had Ct value of more than 37 and 25 samples did not show any amplification.

As per the manufacturers guidelines Ct value of ≥ 37 was considered as negative.

Table 5- Ct values of RT-PCR positive cases

	No of samples with Ct value >20 and <30	**No of samples with Ct value >30 and <37**	**Total**
ICT-Positive RT-PCR-Positive	28 (82 %)	6 (18 %)	34
ICT-Negative RT-PCR-Positive	1 (3%)	29 (97 %)	30

Table 5 shows that out of 64 samples positive by RT-PCR, 34 samples were positive by ICT also. 28(82%) of these samples had Ct values of less than 30. Only 6(18%) samples had Ct value of more than 30. Where as in samples which are negative by ICT and positive by RT-PCR , 29(97%) had Ct values more than 30 and 1(3%) had Ct value of less than 30.(Fig 5)

Fig 4 Ct values of samples tested by RT-PCR

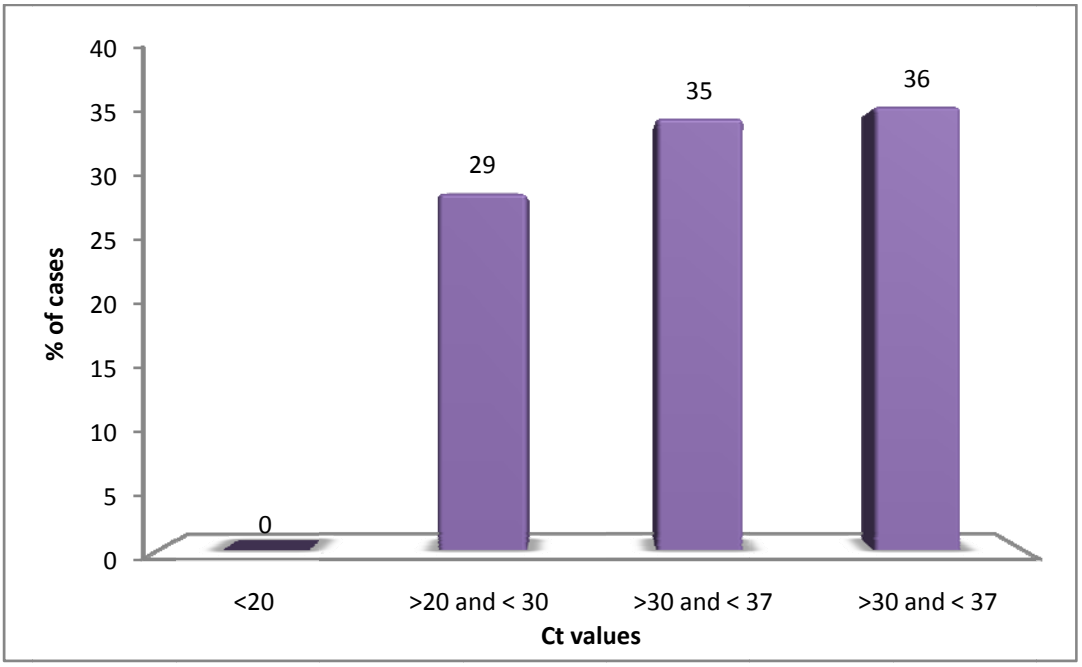


Fig 5 Ct values of RT-PCR positive and negative cases

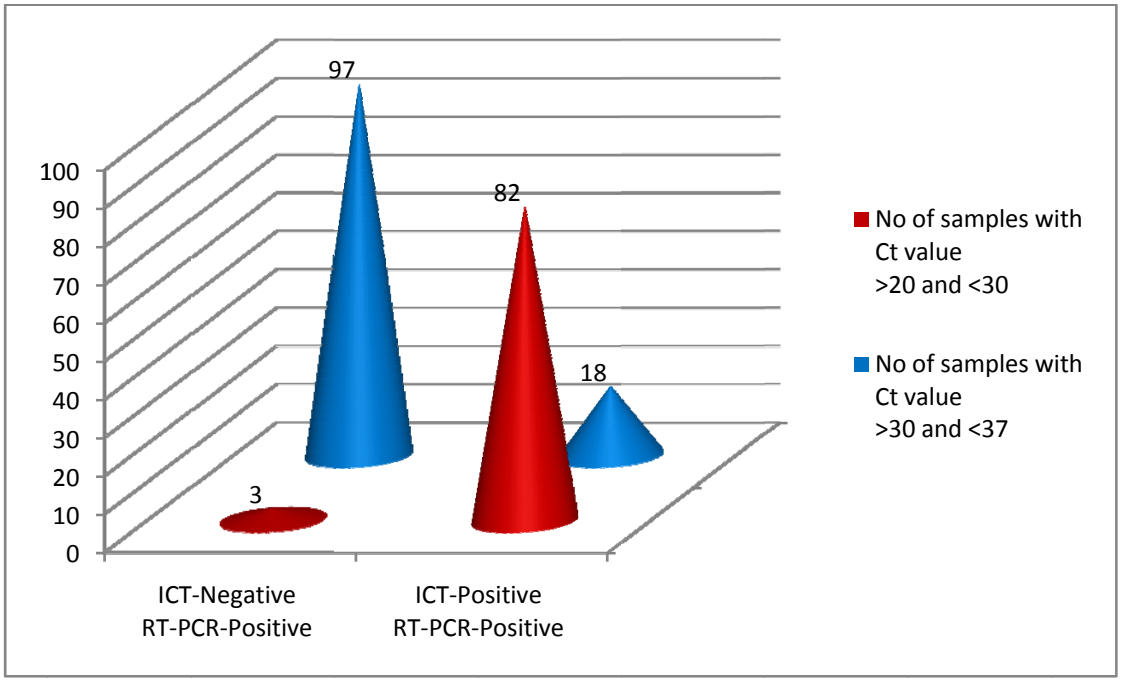


Table-6.Evaluation of rapid ICT against RT-PCR

ICT	RT-PCR	
	Positive	**Negative**
Positive	34	0
Negative	30	36
Total	64	36

The rapid immunochromatographic card test was evaluated for its sensitivity and specificity against RT-PCR ,a gold standard test.

Sensitivity = $\frac{TP}{TP+FN} = \frac{34}{64}x100$ = 53.1%

Specificity = $\frac{TN}{TN+FP} = \frac{36}{36}x100$ = 100%

Positive predictive value = $\frac{TP}{TP+FP} = \frac{34}{34}x100$ = 100%

Negative predictive value = $\frac{TN}{TN+FN} = \frac{36}{66}x100$ = 54.5%

From the above table, sensitivity of rapid ICT was 53.1% when evaluated against RT-PCR, a gold standard test. Specificity was 100 % compared to RT-PCR and positive predictive value of ICT was also 100%.Negative predictive value of ICT was 54.5%.

Table 7. Agewise distribution of rotavirus positive cases

Age group (months)	Positive cases	
	Number	Percentage
0-6	13	20
7-12	39	61
13-18	10	16
19-24	2	3
Total	64	100
Mean	9.5	

The above table shows that majority of positive cases are in the age group of 7-12 months. Out of 64 samples tested by RT-PCR 39 (61%) were in the age group of 7-12 months.Association of this age group with rotavirus positivity was statistically significant.This is followed by a higher incidence in the age group of 0-6 months (20%).(Fig 7)

Table 8 Sexwise distribution of rotavirus positive cases

Sex	Positive cases	
	Number	Percentage
Male	34	53
Female	30	47
Total	64	100

Sexwise distribution of rotavirus positive cases in the above table shows that male and female children are affected almost equally by rotavirus (Fig8) .Out of 64 children positive by PCR, 34 (53%) were male and 30 (47%) were female. There was no difference in gender related to rotavirus infection.

Fig 7 Agewise distribution of rotavirus positive cases

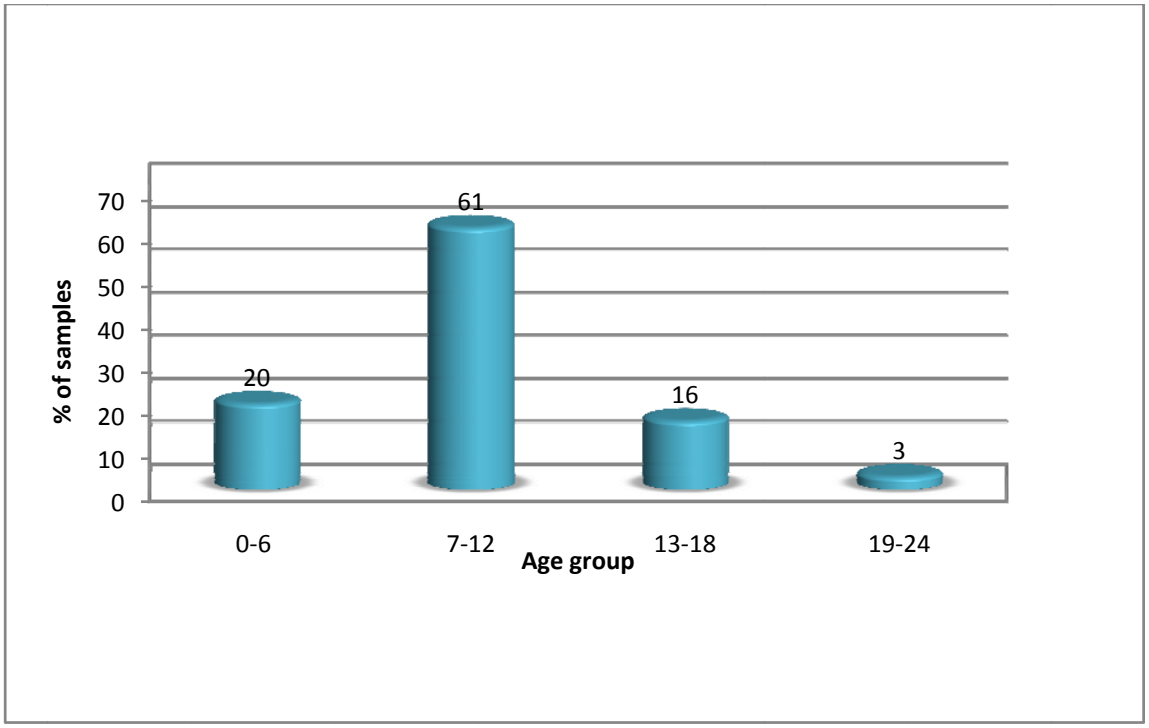


Fig 8 Sexwise distribution of rotavirus positive cases

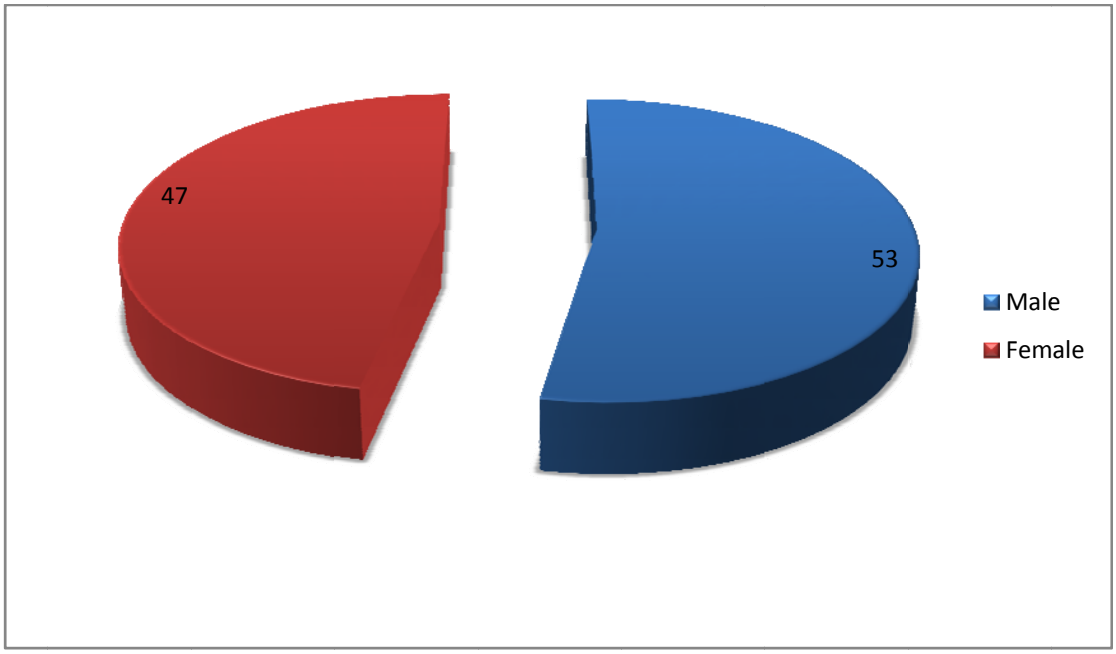


Table-9. Association of urban and rural area in positive cases.

	Positive		Negative		Total	
	No	**%**	**No**	**%**	**No**	**%**
Urban	39	61	19	53	58	58
Rural	25	39	17	47	42	42
Total	64	100	36	100	100	100

The above table shows that out of 64 PCR positive cases, 39(61%) cases belonged to urban area and 25 (39%) to rural area. In PCR negative cases, 19(53%) cases lived in urban and 17 (47%) in rural area.(Fig 9)The association was statistically not significant($p>0.05$).

Table-10. Association of Breast feeding among positive cases.

Breast feeding	Positive cases		Negative cases		Total	
	No	%	No	%	No	%
Breast fed	27	42	23	64	50	50
Bottle fed	37	58	13	36	50	50
Total	64	100	36	100	100	100

The association of breast feeding with rotavirus positive cases was analysed in the above table. In 64 PCR positive children , 27(42%) were breast fed while 37(58%) were not. Among 36 PCR negative children, 23(64%) were breast fed and 13(36%) were not breastfed (Fig 10). It was found that breast feeding had a significant correlation with rotavirus positive and negative children($p<0.05$).

Fig 9 Association of urban and rural area in positive cases

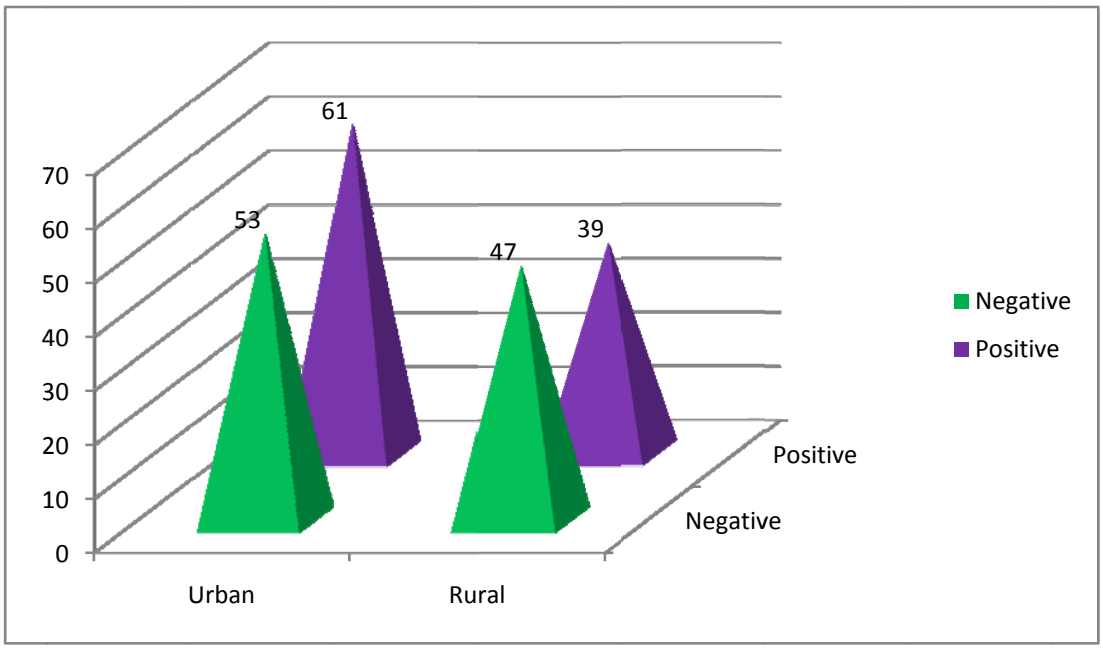


Fig 10 Association of Breast feeding among positive cases

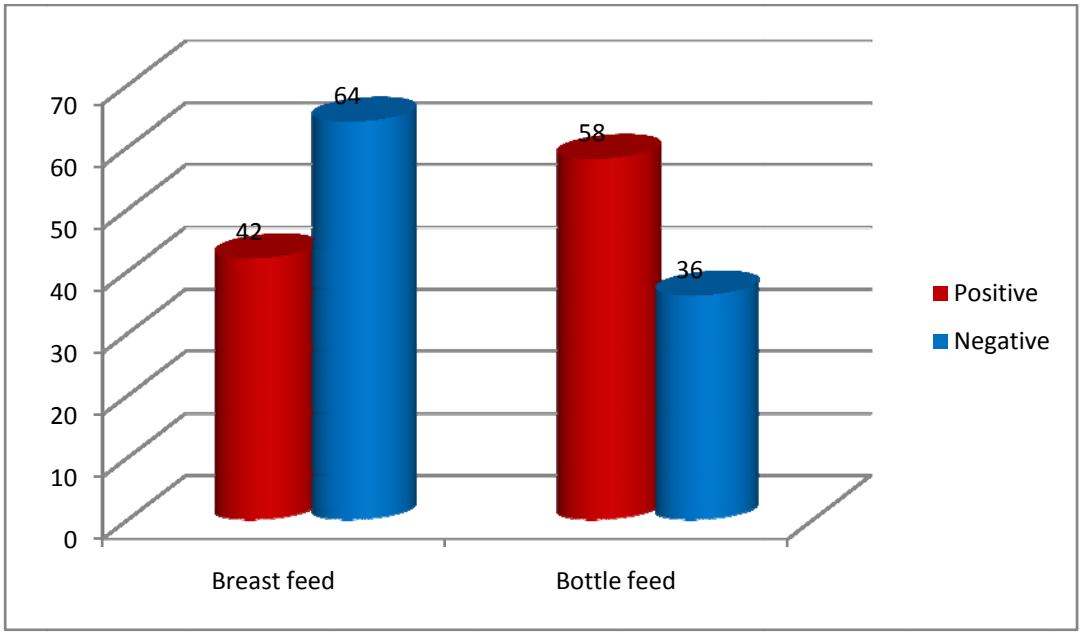


Table 11. Association of vomiting among positive cases

Vomiting	Positive cases		Negative cases		Total	
	No	%	No	%	No	%
Present	52	75	12	39	64	64
Absent	17	25	19	61	36	36
Total	69	100	31	100	100	100

The above table shows that vomiting was an associated feature in 52(75%) of 64 rotavirus positive cases and 12 (39%) of negative cases. Vomiting was absent in 17(25%) of PCR positive cases and 19 (61%) of PCR negative cases.(Fig 11)There was a significant correlation between vomiting and rotavirus positive children. ($p<0.05$)

Table 12. Association of fever among positive cases

Fever	Positive cases		Negative cases		Total	
	No	%	No	%	No	%
Present	43	67	19	53	62	62
Absent	21	33	17	47	38	38
Total	64	100	36	100	100	100

Association of fever in positive cases is shown in the above table.Among 64 PCR positive cases, 43(67%) complained fever as an associated feature while 21(33%) were afebrile. In 36 PCR negative cases, 19(53%) had fever and 17(47%) were afebrile.(Fig 12) Though the percentage of rotavirus positive children with fever was more than that of rotavirus negative children ,the difference was statistically not significant($p>0.05$).

Fig 11 Association of vomiting among positive cases

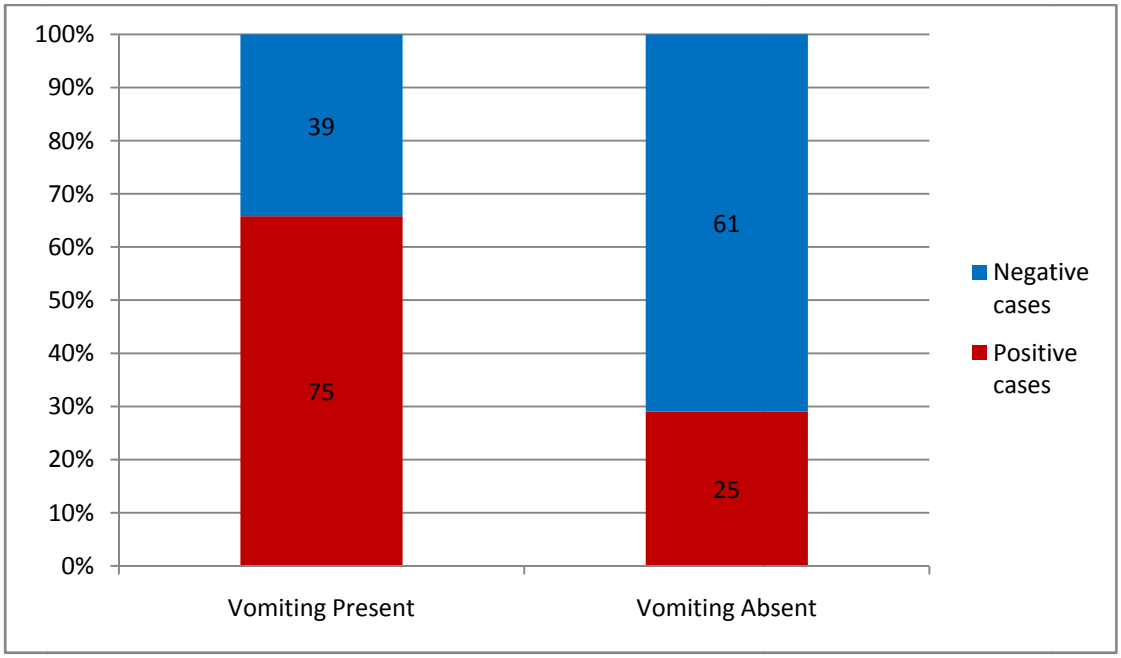


Fig 12 Association of fever among positive cases

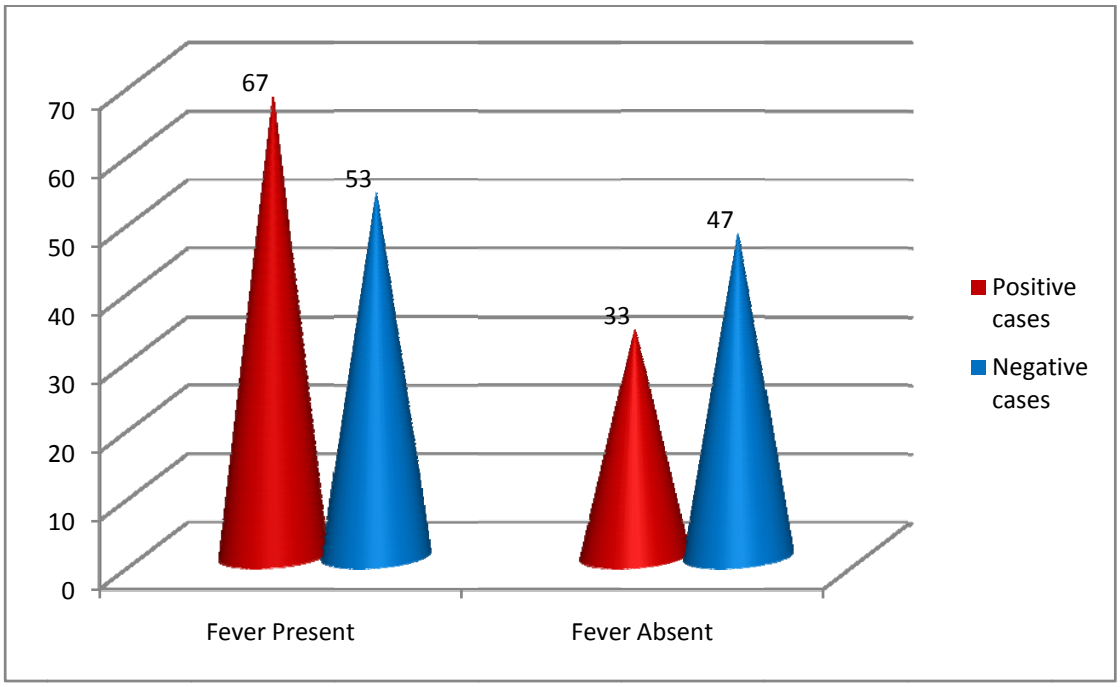


Table 13. Degree of dehydration among positive cases

Dehydration	Positive cases	Negative cases	Total
Nil	17(26%)	25(69 %)	40
Mild	14(22%)	9 (25 %)	25
Moderate	15(23%)	1 (2%)	15
Severe	18(28%)	1 (2%)	20
Total	64	36	100

The above table shows that out of 100 samples tested by RT-PCR, 25 (69 %) of rotavirus negative cases did not have any dehydration and 9 (25%) patients had mild dehydration. Only one (2%) case had moderate and severe dehydration . Among 64 PCR positive samples, 17 (26%)cases were not dehydrated while 14(22%), 15(23%), 18(28%) cases had mild,moderate and severe dehydration respectively. The degree of dehydration was significantly associated with rotavirus positivity ($P<0.001$).

Table 14. Evaluation of vaccine in positive cases

Vaccine	Positive cases		Negative cases		Total	
	No	%	No	%	No	%
Received	0	0	2	6	2	2
Not received	64	100	34	94	98	98
Total	64	100	36	100	100	100

As per the above table ,out of 64 PCR positive children,none had been vaccinated against rotavirus. In 36 PCR negative cases, only 6% (2) of cases had received rotavirus vaccine.(Fig 14) As only 2 children had received rotavirus vaccine among all 100 cases, the association was found to be not statistically significant. The negligible vaccinated subjects did not show any significant inferences ($p>0.05$).

Fig 13 Degree of dehydration in positive cases

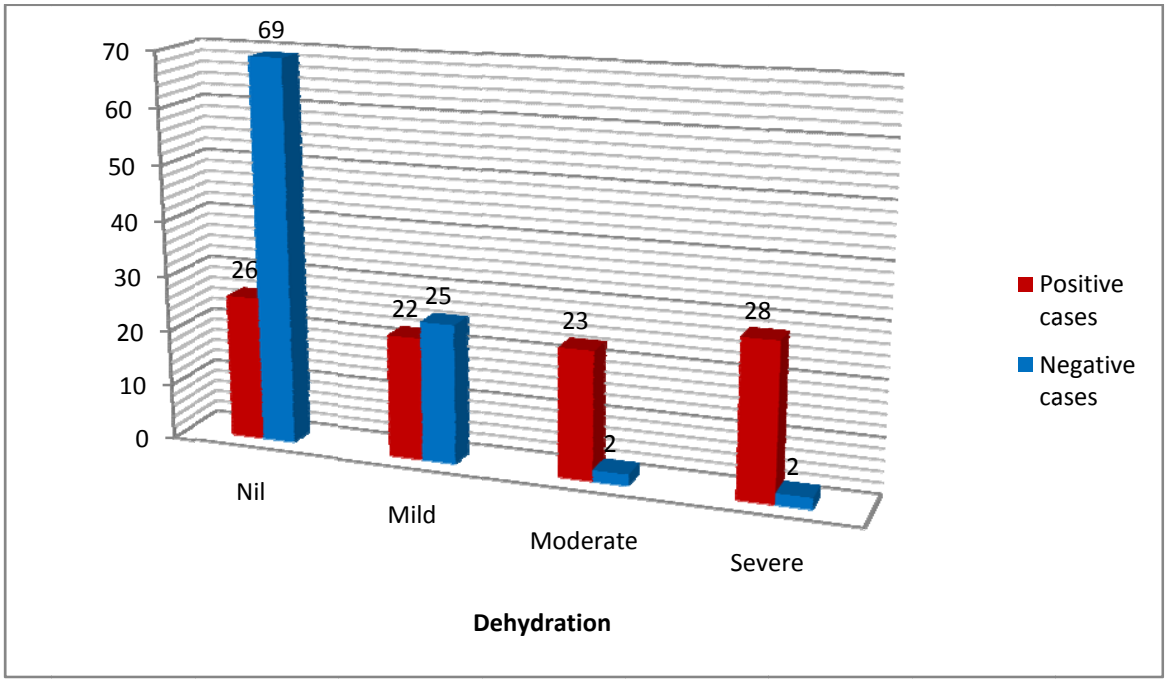
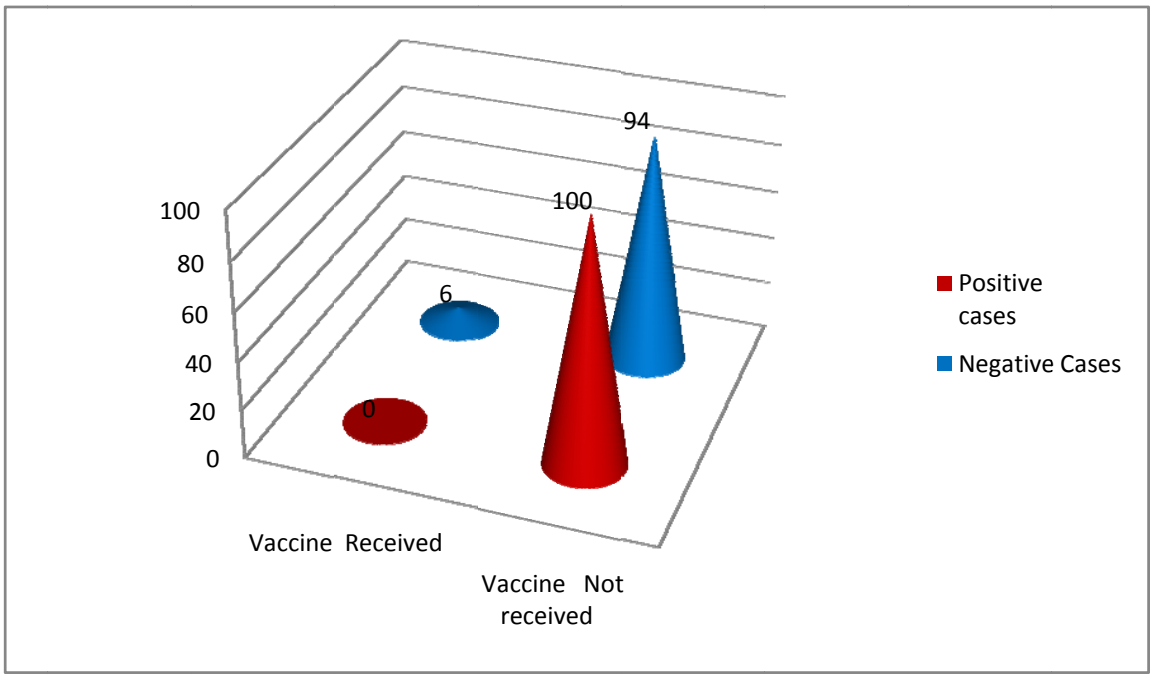


Fig 14 Evaluation of vaccine in positive cases



6. DISCUSSION

Rotaviruses are the most common cause of acute diarrhoeal diaease in young children leading to dehydration.Though morbidity due to rotavirus is similar in both developed and developing countries,mortality is pronounced in developing countries like India.Diagnosis of this infection in children with diarrhoea will help to estimate the real burden of gastroenteritis attributable to rotavirus.Identification of rotavirus by a method which is both sensitive and cost effective will improve the laboratory diagnosis. In this backround this study attempts to evaluate the rapid immunochromatographic card test against RT-PCR in diagnosing rotavirus in stool samples of children less than two years of age.

Stool samples were collected from 100 hospitalized children of less than 2 years of age with diarrhoea over a period of one year.

6.1 Age and sex distribution of children with diarrhoea in the study group

Around half of the patients (57%) in the study group were between 7-12 months of age. This was similar to a study by Calistus Wilunda *et al*.[77] Higher risk of diarrhoea in children aged 6-23 months could be related to beginning of environmental exposure and weaning of children who have immature immune system.Male (51%) and female (49%) children in the study group were equally affected by acute diarrhoeal

diaeases.Gender difference was not associated with the incidence of acute diarrhoea which was similar to a study by Ali Asghar Kolahi *et al*,since boys and girls were probably equally exposed to the risk factors with acute diarrhoea which may be related to the environment,sociodemographic status and other biological agents.[78] In contrast higher incidence of diarrhoea in male children was reported in a study by Calistus Wilunda *et al* which may be due to the increased attention towards the male child.[77]

6.2 Presence of associated clinical features

In this study among all 100 children with diarrhoea,69 (69%) cases had vomiting and 62 (62%) had fever.Lesser number of children were reported with upper respiratory tract infection. Vomiting ,fever,abdominal pain and symptoms suggestive of dehydration are the associated clinical features usually. Vomiting and fever in any case of diarrhoea may worsen the clinical picture by increasing the level of dehydration and decreasing the oral intake.

6.3 Comparison of rapid ICT and RT-PCR in detection of rotavirus

The positivity rate of rapid ICT was 34% while it was 64% by RT-PCR. In the present study rota virus accounts for 64% of hospitalization in children with acute diarrhoeal illness .The prevalence is comparable to a study conducted by Hamsa T Tayeb *et al* in 2011.[23]The increased

incidence of rotaviral diarrhoea in this study may be partly due to the method by which they are tested,RT-PCR ,a highly sensitive one.The amount of severe gastroenteritis caused by rotavirus has increased from an average of 25% in studies which were completed prior to 2000 to over 38% in studies that were completed after 2005.[32] It is postulated that improvements in sanitation and use of antimicrobials have had a greater impact on preventing bacterial and parasitic gastroenteritis (GE) than rotavirus .

6.4 Ct values of samples tested by RT-PCR

Ct values of all the samples tested were in the wide range of 20-40 . The detection rates will be improved by installing Ct cutoff values in RT-PCR giving a rapid and relevant diagnosis.

6.5 Ct values of RT-PCR positive and negative cases

Of the 30 that were PCR positive and ICT negative, the Ct values were high (≥30), indicating a low viral load . Correlation of Ct value with viral load as evidenced by Petra F. G. Wolffs *et al* reinforces that higher Ct values are associated with low viral load.[60]

6.6 Evaluation of rapid ICT against RT-PCR

The sensitivity of ICT was low (53.1%) while the specificity was 100% when evaluated against RT-PCR. Similar low sensitivity was reported by Petra F. G.*et al*. In this study , true positive rates of the test

was 75% and the ability to exclude false positives was 95% for rotavirus.[60] Hence all the samples negative by ICT have to be confirmed by a more sensitive test like ELISA or PCR.

6.7 Agewise distribution of rotavirus positive cases

Prevalence of rotaviral diarrhoea was more common in the age group of 7-12 (61%) in this study .Similar finding was seen in a study done by M Kargar *et al* [38] and Vivek Jain *et al* [79] . The higher incidence in 7-12 months was followed by the age group 0-6 months (20%).This may be due to breast feeding ,exclusive or partial in the first 6 months and also due to the acquired immunity from the mother which may prevent infection in the first few months of life. The lowest incidence of rota virus gastroenteritis in children over 18 months of age(3%) might be due to acquired immunity from previous infection either symptomatic or asymptomatic.

6.8 Sexwise distribution of positive cases

Present study revealed no association of sex with the incidence of rotavirus infection .This is in contrast to the study by Kelkar SD *et al*[26] and Trung Vu Nguyen *et al* [43] where male predominance was noted. Whether this was incidental or due to more care for the male child has to be studied. A higher female incidence was noted in a study by Sherchand JB *et al* in Nepal.[44]

6.9 Association of urban / rural area among positive cases

There was no significant difference between the incidence of rotavirus infection in children living in rural and urban areas.Similar results have been reported by V. Mishra *et al* in northern India.[45] In a Turkish study it was found that rotavirus infection is not affected by socioeconomic and educational status of parents .[46]

6.10 Impact of breast feeding in positive cases

There was a significant difference in the incidence of rota virus gastroenteritis among breast fed and non breast fed babies .64 % of children who were negative for rotavirus were breastfed implying that breast feeding was effective in reducing the frequency of rota virus gastroenteritis. Plenge-Bönig A *et al* [80] and Indrani Banerjee *et al* [31] also reported a stronger protective effect of breast feeding against rotavirus infection.This is because human milk contains human rota virus specific IgA antibodies and these antibodies are capable of neutralizing rota virus antigens . Human milk contains a 46 kDa mucin-associated glycoprotein, lactadherin, which binds specifically to rotavirus and inhibits its replication.[81]Breastfeeding is important to diminish rotavirus-related gastroenteritis in infants until they are vaccinated. Breast feeding also has a role in reducing the hospital stay and mortality due to rotaviral diarrhoea.

6.11 Association of vomiting among positive cases

In this study vomiting was more frequent among positive cases (75%), than among children whose samples were negative (39%) for virus .This was similar to the studies by I Uhnoo *et al* [36] , Bon *et al* [82] and M Kargar,*et al* [38] .Occurrence of vomiting in the course of the illness is explained by invasion of the upper part of small intestine with rota virus. Blacklow *et al* [83] have shown that rota virus gastroenteritis is accompanied by abnormal gastric motor function , and this abnormality may be the cause of vomiting. Presence of vomiting will reduce the intake of oral rehydration solution and lead to further deterioration of the clinical condition.

6.12 Association of fever among positive cases

There was no difference in fever incidence between rotavirus positive and negative cases. Though fever was present in 67 % of positive cases, the association was not significant since 53% of negative cases also had fever. This may be due to the presence of other infective causes in rotavirus negative samples.

This was similar to the studies by M. El-Mougi *et al* [84] and Bon *et al* [82].According to CDC up to one-third of children with rotavirus infection may be febrile (greater than 102°F). M Kargar *et al* [38] showed

that children with rotavirus infection had diarrhoea with fever (52.08%) which is in contrast to the present study.

6.13 Level of dehydration among positive cases

In this study 69% of negative cases did not suffer from any dehydration while in the positive cases around 50% cases suffered from moderate to severe dehydration.Similar report was given by Gururaj Aithala *et al* in which most of the affected infants and children had mild to moderate dehydration. [85]

6.14 Status of vaccination among positive cases

Out of 100 children included in the study only two had received rotavirus vaccine.As the number of vaccinated children is negligible,the protective efficacy of vaccine could not be assessed.This reflects that though rotavirus vaccine has been introduced in India in 2006, there is a huge part of deserving susceptible children have not received the vaccine.Inclusion of rotavirus vaccine in UIP may achieve a reduction in morbidity and mortality due to rotavirus . After RV1 introduction, Mexico saw a 35% reduction in the rate of diarrhoeal deaths among children age appropriate for the vaccine [86]. After RV1 introduction in Brazil in 2006, 30% and 39% decreases in gastroenteritis mortality were noted in 2007 and 2008, respectively, when compared to the mortality rates in 2004-2005.[87]

Active surveillance with sensitive methods like PCR in rotavirus case finding along with further strain typing in an area will help to formulate a vaccine according to the prevalence in that area.Including the vaccine in UIP and strict adherence to vaccination schedule will significantly reduce the disease burden due to rotavirus infection in children less than 2 years in our country, as India is the leading cause of rotaviral diarrhoeal diaease in the world.

7. SUMMARY

The present study aimed at detecting the prescence of rotavirus in stool samples of children less than two years of age ,admitted for diarrhoea. 100 cases of hospitalized children were included in the study and samples were tested by both rapid immunochromatographic test and RT-PCR.The rapid card test was evaluated against RT-PCR in detection of rotavirus in stool samples.The factors associated with rotaviral diarrhoea in children were analysed.

- Of the 100 children tested 55 % of children with acute diarrhoeal disease belonged to the age group of 7-12 months.
- 51% were male and 49% were female in the study group.
- Vomiting,Fever and URI were the associated clinical features in children with diarrhea.
- Out of 100 children tested by rapid ICT, 34% were positive for rotavirus antigen in the stool sample.
- When the same samples were tested by RT-PCR ,positivity was 64%.
- All 34 samples positive by ICT were positive by RT-PCR also.
- Out of 64 positive samples 29 had Ct values of 20-30 and 35 samples had Ct values of 30 -37.

- Of the 30 samples which were negative by ICT and positive by RT-PCR,29 samples had higher Ct value of >30 <37.
- Rapid ICT had a sensitivity of 53.1%, Specificity of 100% when evaluated against RT-PCR as gold standard.
- The positive predictive value of ICT was 100% and the negative predictive value was 54.5 %.
- Maximum number of positive cases (69%) occurred in the age group of 7-12 months.
- There was no gender difference related to rotavirus infection.
- No significant difference in the rotavirus infection was observed between the children living in urban or rural area.
- Most of the positive cases (58%) were not breast fed while 64% of negative cases were breastfed.
- Vomiting was present in 75% of rotavirus positive cases.
- Fever was observed in 67% of positive cases.
- More than 50% of positive cases had moderate to severe dehydration.
- Out of 100 children tested only 2 had received rotavirus vaccine who were negative for rotavirus.

8. CONCLUSION

- The present study revealed that rotavirus is the major cause of acute diarrhoea in infants and children less than two years of age.
- So,all children with acute gastroenteritis should have their stool examined for rota virus for purpose of correct diagnosis to avoid unnecessary use of antibiotics and emergence of drug resistance.
- Though ICT has lower sensitivity than PCR ,it can be used as a alternate test for rapid diagnosis in situations of emergency testing,as an outpatient procedure.
- Highly sensitive detection method like RT-PCR is necessary to find out the exact number of cases with rotavirus infection.
- Further molecular studies are essential to know the accurate information of rotavirus serotypes which will be helpful in formulating vaccines in future.

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ANNEXURE –I

(Proforma of the Data sheet)

DATA SHEET FOR COLLECTION OF SOCIO DEMOGRAPHIC, CLINICAL AND LABORATORY DATA

FOR P.G. DISSERTATION WORK ON

COMPARISON OF IMMUNOCHROMATOGRAPHY WITH RT-PCR FOR DETECTION OF ROTAVIRUS IN FECAL SAMPLES

Name: Age /Sex:

IP/OP no:

Address:

Rural/urban Date:

Informant:

Present complaints:

1.Loose stools

Onset	Duration	Frequency	Blood	Mucus

2.Vomiting

YES	NO

3.Fever

YES	NO

4. URI

YES	NO

5.Level of dehydration : Nil / Mild /Moderate / Severe

6.H / O Rotavirus vaccination:

7.H/ O Breast feeding:

TREATMENT

INVESTIGATIONS:

Rapid Immunochromatography:

RT-PCR:

Data Sheet of Socio Demographic, Clinical, Laboratory results of Comparison of immunochromatography with RT-PCR for detection of Rotavirus in fecal samples

Serial no	Age	Sex	ICT Adeno	ICT Rota	PCR CT	PCR result	vomiting	fever	URI	dehydra	AMA	IVF	Urban	BF	vaccine
1	12	male	N	N	33	P	N	P	N	mild	Yes	Yes	R	N	N
2	12	female	P	N	39.29	N	P	N	N	mild	Yes	Yes	R	N	N
3	10	female	P	N	35.66	P	P	N	N	No	Yes	No	R	Y	N
4	6	female	N	N	35	P	N	N	N	No	Yes	No	R	Y	N
5	18	female	N	N	38.41	N	N	P	P	No	Yes	No	U	Y	N
6	12	male	N	P	28.13	P	P	P	N	mode	Yes	Yes	U	N	N
7	9	male	N	N	30.45	P	P	P	N	severe	Yes	Yes	R	N	N
8	6	male	N	N	33	P	N	N	N	No	Yes	No	U	Y	N
9	8	female	N	P	24	P	P	P	N	mild	Yes	Yes	U	N	N
10	7	male	P	N	35.74	P	P	N	N	No	Yes	No	R	Y	N
11	8	female	N	N	39.2	N	P	P	P	mild	Yes	No	R	Y	N
12	12	male	N	P	23.56	P	P	P	N	severe	Yes	Yes	U	N	N
13	12	male	N	P	24.9	P	P	P	N	mode	Yes	Yes	R	N	N
14	4	male	N	P	20.31	P	P	P	N	mode	Yes	Yes	U	Y	N
15	7	female	N	P	30.34	P	P	P	N	mild	Yes	No	U	Y	N
16	3.5	male	N	N	34.97	P	N	N	N	No	No	No	U	Y	N
17	6	male	N	P	24.9	P	N	P	P	mild	Yes	Yes	U	Y	N
18	15	male	N	N	34.89	P	N	N	N	No	Yes	No	R	N	N
19	6	male	N	P	31.52	P	P	P	N	severe	Yes	Yes	R	Y	N
20	15	female	N	N	35.26	P	N	N	N	No	Yes	No	U	N	N
21	9	male	N	N	39.13	N	N	N	N	No	No	No	U	Y	N
22	9	male	P	N	39.02	N	P	P	N	No	Yes	No	U	Y	N
23	7	female	N	N	0	N	N	P	P	No	Yes	No	R	Y	N
24	8	female	P	N	0	N	N	N	N	No	No	No	R	Y	N
25	6	female	N	N	0	N	N	N	N	No	No	No	R	Y	N
26	15	male	N	N	35.93	P	N	N	N	No	No	No	R	N	N
27	11	male	N	P	25.21	P	P	P	N	mode	Yes	Yes	R	N	N
28	8	male	N	N	36.92	N	P	P	N	severe	Yes	Yes	U	Y	N
29	9	female	N	P	24.4	P	P	P	N	severe	Yes	Yes	U	N	N
30	3	male	N	P	26.61	P	P	P	P	severe	Yes	Yes	R	N	N

31	12	female	N	N	39.14	N	N	N	N	No	Yes	No	R	N	N
32	9	male	N	N	32.02	P	P	P	N	mode	Yes	Yes	U	N	N
33	5	male	N	N	33.14	P	P	N	P	mild	No	Yes	R	Y	N
34	15	female	N	P	29.47	P	N	P	N	mild	Yes	Yes	R	N	N
35	9	female	N	P	27.42	P	P	P	N	mode	Yes	Yes	U	Y	N
36	15	male	N	N	39.42	N	P	P	N	mild	Yes	Yes	U	N	N
37	11	female	N	N	33.5	P	N	N	N	No	Yes	No	U	N	N
38	11	male	N	P	30.19	P	P	P	N	mode	Yes	Yes	U	N	N
39	12	male	N	N	34.8	P	P	P	N	mode	Yes	Yes	U	N	N
40	8	female	N	P	29.86	P	P	P	N	severe	Yes	Yes	R	Y	N
41	12	female	N	N	33.45	P	P	P	N	mild	Yes	Yes	U	N	N
42	10	female	P	N	35.14	P	P	P	N	mild	Yes	Yes	R	N	N
43	7	female	N	P	30.46	P	P	N	P	mild	Yes	Yes	U	Y	N
44	6	male	N	P	25.05	P	P	P	P	mode	Yes	Yes	U	Y	N
45	9	male	N	N	33.19	P	P	P	N	severe	Yes	Yes	R	Y	N
46	10	female	N	P	29.35	P	P	P	N	severe	Yes	Yes	U	N	N
47	5	female	N	P	29.49	P	P	P	P	severe	Yes	Yes	R	Y	N
48	18	female	N	P	28.38	P	P	P	N	severe	Yes	Yes	U	N	N
49	11	male	N	P	26.19	P	P	P	N	severe	Yes	Yes	R	N	N
50	12	female	N	P	28.26	P	P	P	N	mode	Yes	Yes	U	N	N
51	11	male	N	N	34.35	P	N	N	N	No	Yes	Yes	U	Y	N
52	5	male	N	N	31.97	P	P	P	N	mode	No	Yes	U	Y	N
53	10	female	N	P	23.16	P	P	P	N	mode	Yes	Yes	U	Y	N
54	15	male	N	N	31.91	P	P	N	N	No	Yes	Yes	U	N	N
55	8	male	N	P	30.15	P	N	N	N	No	Yes	No	U	Y	N
56	11	female	N	P	25.01	P	P	P	N	severe	Yes	Yes	U	N	N
57	12	male	N	P	30.8	P	P	P	N	No	Yes	Yes	U	N	N
58	15	female	N	N	34.45	P	P	P	N	mode	Yes	Yes	U	N	N
59	11	female	N	P	25.52	P	P	P	N	mode	Yes	Yes	R	N	N
60	10	male	N	N	31.19	P	P	P	N	severe	Yes	Yes	U	Y	N
61	15	female	N	P	29.24	P	P	N	N	mild	Yes	Yes	U	N	N
62	12	male	N	N	30.68	P	P	P	N	severe	Yes	Yes	R	N	N
63	4	female	N	N	33.13	P	P	N	N	No	No	No	R	Y	N
64	12	male	P	N	37.64	N	N	N	P	No	Yes	No	R	N	N
65	18	female	N	N	0	N	P	P	N	mild	Yes	Yes	U	N	N

66	8	female	N	P	25.78	P	P	P	N	severe	Yes	Yes	U	Y	N
67	15	male	N	N	34.55	P	P	P	N	No	Yes	Yes	U	N	N
68	11	female	N	N	35.62	P	P	N	N	No	Yes	Yes	U	Y	N
69	3	male	P	N	37.32	N	N	N	N	mild	Yes	Yes	U	Y	N
70	5	female	N	N	37.79	N	P	P	P	No	Yes	No	U	Y	N
71	7	male	N	P	24.66	P	P	P	N	severe	Yes	Yes	U	Y	N
72	12	female	N	N	34.7	P	P	N	N	mild	Yes	Yes	R	N	N
73	21	female	N	N	32.32	P	P	P	N	mild	Yes	Yes	U	N	N
74	15	male	N	P	29.77	P	P	N	N	No	Yes	Yes	U	N	N
75	12	female	N	N	31.34	p	P	N	N	mild	Yes	Yes	U	N	N
76	11	male	N	N	0	N	N	P	N	No	Yes	No	R	Y	N
77	3	female	N	N	0	N	P	N	N	No	Yes	No	R	Y	N
78	10	male	N	N	0	N	P	P	P	mode	Yes	Yes	R	Y	N
79	8	female	N	N	0	N	N	N	N	No	No	No	R	Y	N
80	9	male	N	P	29.29	P	P	P	N	severe	Yes	Yes	U	Y	N
81	5	female	N	N	0	N	P	N	N	No	No	No	U	N	N
82	10	male	N	N	0	N	N	P	N	No	Yes	Yes	U	Y	Y
83	12	male	N	N	0	N	N	P	P	No	Yes	Yes	R	N	N
84	7	male	N	P	24.76	P	P	P	N	severe	Yes	Yes	R	N	N
85	18	female	N	N	0	N	P	P	N	mild	Yes	Yes	R	Y	N
86	5	female	N	N	0	N	P	N	N	No	No	No	R	N	N
87	5	female	N	N	0	N	N	N	N	No	No	No	R	Y	N
88	7	female	N	N	0	N	P	N	N	mild	Yes	Yes	U	Y	N
89	18	male	N	N	0	N	N	P	N	No	Yes	No	U	N	N
90	4	female	N	P	28.6	P	P	N	N	mild	No	Yes	R	Y	N
91	15	male	N	N	0	N	N	P	N	No	Yes	No	U	N	N
92	12	male	N	N	0	N	N	P	N	No	Yes	No	U	N	N
93	18	female	N	N	0	N	P	P	N	mild	Yes	No	U	N	N
94	2	male	N	N	0	N	P	N	N	No	No	No	U	Y	N
95	3	male	N	N	0	N	P	N	N	No	No	No	U	Y	N
96	9	female	N	N	0	N	P	P	P	mild	Yes	Yes	U	Y	N
97	24	female	N	N	0	N	N	P	N	No	Yes	No	R	N	Y
98	21	female	N	N	26.31	P	P	P	N	mode	Yes	Yes	R	N	N
99	5	male	N	N	0	N	N	N	N	No	Yes	No	U	Y	N
100	4	male	N	N	0	N	N	N	P	No	No	No	R	Y	N